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15. SUPPLEMENTARY NOTES

14. ABSTRACT In this program project, 72 brains were examined, including 32 brains of subjects with idiopathic autism (unknown etiology), 12 brains of individuals with autism associated with chromosome15 duplication (dup15) and 28 brains of control subjects from 2 to 65 years of age. The study revealed several correlations between structural and biochemical changes and autistic phenotype.

1. The deficit of neuron volume detected in all 16 brain structures and 15 of 19 of their anatomical subdivisions in 4-8 year old children with autism is an indicator of global developmental encephalopathy in autism of unknown origin. Brain region-specific index of neuronal volume deficit is a sign of desynchronized development of anatomically and functionally related neurons that may explain social and communication deficits, and restricted repetitive and stereotyped patterns of behavior. Reduction of the developmental deficit from on average 19.6% in 4-8 year old to 8.8% in >8 year old subjects, indicates delayed acceleration of growth of neurons in late childhood and adulthood. The study expands corticocentric theory with evidence that autism is associated global developmental encephalopathy with delayed and desynchronized neuron growth in cortical and subcortical gray matter.

2. Abnormalities of Aβ intracellular accumulation in early stage of brain development suggest their link to the clinical phenotype, including seizures, in idiopathic autism and autism associated with idic15. Aβ accumulation in neurons initiates oxidative stress resulting in lipids peroxidation. Accumulation of Aβ in activated astrocytes results in their death. Both are markers of developmental alterations in APP processing with structural and functional consequences.

3. Severe developmental abnormalities in the flocculus combined with reduced volume of Purkinje cells in the entire cerebellum of autistic subjects but no changes in morphology and neuronal size in the inferior olive, the second component of the olivo-floccular integrator of oculomotor function, indicates that mechanism leading to floccular dysplasia play a pivotal role in defective function of the oculomotor system in autism and abnormal gaze.

Table of Contents
Subproject 1 (PI: Jerzy Wegiel)
Annual Report

Page

Annual Report #4
August 28, 2012

Program Project Title: Characterization of the Pathological and Biochemical Markers that Correlate to the Clinical Features of Autism

Program Project PI: Jerzy Wegiel, Ph.D.; Co-PI: W. Ted Brown, M.D., Ph.D.

The overall aim of this multidisciplinary program project is to establish correlations between morphological and biochemical markers of autism and the clinical symptoms of the disorder.

SUBPROJECT 2

Contribution of significant delay of neuronal development and metabolic shift of neurons to clinical phenotype of autism

Subproject 2 P.I.: Jerzy Wegiel, Ph.D.

INTRODUCTION

The overall aim of this multidisciplinary program project is to establish correlations between morphological and biochemical markers of autism and clinical symptoms of disease. To achieve these goals, we proposed three subprojects. The factor integrating these three closely collaborating groups is the concentration of a broad spectrum of aims and methods on brains of 72 subjects including: 32 brains of autistic people, 12 brains of individuals with autism associated with chromosome15 duplication (dup15) and 28 brains of control subjects.

This Program Project is focused on the detection of:
(a) mechanisms leading to morphological changes and the clinical autism phenotype,
(b) morphological and biochemical markers of autism,
(c) correlations between pathology and clinical manifestations of autism, and
(d) those pathological domains that might be a target for treatment.

Progress of work is consistent with the original Program Project and Project 1 aims and timetable.

Material : We examined 72 subjects including: 32 brains of autistic people, 12 brains of individuals with autism associated with chromosome15 duplication (dup15) and 28 brains of control subjects from 2 to 65 years of age. The neuropathological criteria were established in cooperation with Project 1. Cases not meeting the ADI-R criteria and cases with signs of comorbidity, perimortem and postmortem changes affecting brain structure were excluded from the morphometric studies.

Brain structures selected for morphometric study of developing, mature and aging brain of autistic people.
Consistent with the Statement of Work, we examined four brain structures and their subdivisions (for a total of 9 brain subregions), most likely affected by developmental delay and metabolic aberration in adults and aged people with autism:
(1) Amygdala: lateral, latero-basal, accessory basal and central nuclei (aggression, fear, anxiety, memory, cognition).
(2) Caudate nucleus, (3) putamen, (4) globus pallidus (stereotypes, rituals).
(5) N. accumbens ("social brain", reward system).
(6) Nucleus supraopticus and
(7) N. paraventricullaris. These hypothalamic nuclei are (a) the source of numerous growth and trophic factors necessary for normal brain development and function, and (b) the source of factors regulating social memory and attachments, emotional responses, and cognitive functions.
(8) Cerebellum: cortex, white matter (language, pointing, motor functions, cognition).
(9)Dentate nucleus (language, pointing, motor functions, cognition).

However, we found that to achieve a more global view on brain developmental alterations we needed to expand the list of examined brain regions by adding eight brain structures:
(1) Entorhinal cortex (input to the memory system)
(2) Hippocampal formation (memory system; processing and storage of cortical data)
(3) Thalamus (a key component of networks implicated in attention, memory, language, and emotional processing)
(4) Claustrum (integration of function of several brain modalities contributing to cognition)
(5) Substantia nigra (source of dopamine controlling motor, reward and other systems)
(6) Inferior olive (part of the olivo-floccular system controlling eye movement)
(7) Nucleus of facial nerve (facial expression)
(8) Cerebellar flocculus (due to a specific role of the cerebellar flocculus in gaze control, we designed a detailed study of the unique developmental alterations in the flocculus).

After these expansions, the study of a global model of brain developmental abnormalities integrates 17 localized models of developmental alterations in the brain of autistic and control subjects.

Publications (2012)

a. Two papers were published in 2012.
b. Two others were submitted for publication in 2012.
c. One chapter has been submitted in 2012 and one is preparation for publication in 2013.
d. Two other projects are in progress.

Outcome: Historically, this is the largest postmortem morphological, morphometric, and biochemical multidisciplinary study integrating efforts of several groups concentrated on the link between etiology, genetic defects, developmental and age-associated changes of brain structure and metabolism contributing to clinical phenotype of autism (See: Key Research Accomplishments and Conclusions).

Request for no-cost extension. To complete two studies in progress and to respond to reviewers requests, we are asking for a no cost extension of Project 2 and the other two Projects (1-Dr Thomas Wisniewski and 3 – Dr Abha Chauhan).

BODY

This review summarizes results of work on 11 subprojects closing this grant

1. Differences Between the Pattern of Developmental Abnormalities in Autism Associated with Duplications 15q11.2-q13 and Idiopathic Autism

Wegiel J, Schanen CN, Cook EH, Sigman M, Brown WT, Kuchna I, Nowicki K, Wegiel J, Imaki H, Ma SY, Marchi E, Wierzba-Bobrowicz T, Chauhan A, Chauhan V, Cohen IL, London E, Flory M, Lach B, Wisniewski T. J Neuropathol Exp Neurol 2012, 71, 382-397.

See Report #1 (Dr. Thomas Wisniewski PI)

2. Amyloid Abnormal Intracellular Accumulation and Extracellular Aβ Deposition in Idiopathic and Dup15q11.2-q13 Autism Spectrum Disorders

Wegiel J, Frackowiak J, Mazur Kolecka B, Schanen NC, Cook EH, Sigman M, Brown WT, Kuchna I, Wegiel J, Nowicki K, Imaki H, Ma SY, Chauhan A, Chauhan V, Miller DL, Mehta PD, Cohen IL, London E, Reisberg B, de Leon MJ, Wisniewski T. PloS ONE 2012, 7, e35414.

Recent studies indicate that non-amyloidogenic cleavage of the amyloid-β peptide precursor (APP) with α and γ secretases is linked to several developmental disorders, including autism and fragile X syndrome (FXS) (Sokol et al 2006, Westmark and Malter 2007, Westmark et al 2011, Bailey et al 2008, Sokol et al 2011). The proteolytic cleavage of APP by membrane associated secretases releases several Aβ peptides possessing heterogeneous amino- and carboxyl-terminal residues including: $Aβ_{1-40}$ and $Aβ_{1-42}$ as products of β- and γ-secretases (amyloidogenic pathway); $Aβ_{17-40/42}$, as a product of α- and γ-secretases (p3 peptide, non-amyloidogenic pathway) (Iversen et al 1995, Selkoe et al 2001); and $Aβ_{pE3}$ as a product of N-terminal truncation of full length Aβ peptide by aminopeptidase A and pyroglutamate modification (Sevalle et al 2009). Aβ peptides differ in toxicity, oligomerization, fibrillization, distribution and trafficking within cells, and their contribution to Aβ deposits in plaques and vascular walls. Alzheimer disease (AD) is associated with oligomeric Aβ accumulation, fibrillar Aβ deposition in plaques, neuronal degeneration, and cognitive decline. Intraneuronal Aβ accumulation has been shown to be an early event in AD brains, and in transgenic mouse models of AD, that is linked to synaptic pathology (Gouras et al 2010, Bayer and Wirth 2010).

The aims of this comparative study of the brains of subjects with idiopathic autism (autism of unknown etiology) and with a known cause of autism [maternal dup(15)] was to test the hypothesis that regardless of the causative mechanism, autism is associated with an enhanced accumulation of Aβ in neuronal cytoplasm; (b) to show that intraneuronal Aβ is the product of non-amyloidogenic α-secretase APP cleavage ($Aβ_{17-40/42}$); (c) to show brain region and cell type-specific Aβ immunoreactivity; and (d) to identify cytoplasmic organelles involved in Aβ accumulation in the neurons of autistic and control subjects.

Enhanced $Aβ_{17-40/42}$ immunoreactivity is observed in neurons in more than 50% of subjects diagnosed with idiopathic autism. Remarkably, there is a more pronounced Aβ load in the majority of individuals diagnosed with duplications 15q11.2-q13 (dup15) and autism spectrum disorder (ASD), than in idiopathic ASD. This finding suggests early enhancement of APP processing with α-secretase. Aβ accumulation in neuronal endosomes, cathepsin D- and Lamp1-positive lysosomes and lipofuscin, as revealed by confocal microscopy, indicates that enhanced α-secretase processing is paralleled by enhanced proteolytic activity. The presence of $Aβ_{1-40/42}$ in diffuse plaques in three subjects wit ASD, 39 to 52 years of age, suggests that there is an age-

associated risk of metabolic developmental alterations with an intraneuronal accumulation of a short form of Aβ and an extracellular deposition of full length of Aβ in nonfibrillar plaques. The accumulation of $A\beta_{17\text{-}40/42}$ in the astrocytes of some autistic children and adults, and in the plaque perimeter in all three plaque-positive subjects may indicate that the astrocytic cytoplasmic Aβ reflects attempted clearance and partial degradation of full length Aβ by astrocytes. The higher prevalence of Aβ alterations, early onset of intractable seizures, and a high risk of sudden unexpected death in epilepsy (SUDEP) in autistic subjects with dup(15) compared to subjects with idiopathic ASD support the concept of mechanistic and functional links between autism, epilepsy, and alterations of APP processing leading to neuronal and glial Aβ accumulation, and diffuse plaque formation.

3. Contribution of Olivo-floccular Circuitry Developmental Defects to Atypical Gaze in Autism

Wegiel J, Kuchna I, Nowicki K, Imaki H, Wegiel J, Ma SY, Azmitia EC, Banerjee P, Chauhan A, Chauhan W, Cohen IL, London E, Brown WT, Wisniewski T. Submitted to J Autism and Dev Disabilities.

Individuals with autism demonstrate atypical gaze, deficits in facial perception, altered movement perception, and impairments in smooth pursuit (Rosenhall et al 1988; Scharre and Creedon, 1992; Takarae et al 2004). A substantial number of Purkinje cells in the cerebellar flocculus receive converging visual inputs from functionally distinct portions of the retina and subserve the neural mechanisms for oculomotor control during slow eye movements.

The flocculus provides the oculomotor system with eye position information during fixation and with eye velocity information during smooth pursuit (Noda and Suzuki 1979). Our studies indicate that in majority of autistic subjects the flocculus is affected by dysplastic changes (Wegiel et al 2010).

The oculomotor neural integrator circuit requires interactions with oculomotor neurons of the inferior olive nuclei. The presence of olivary dysplasia in three of the five autistic subjects and ectopic neurons related to the olivary complex in two cases (Bailey et al 1998) suggest that oculomotor circuitry is prone to developmental defects.

This study of the inferior olive and the cerebellar flocculus in 12 autistic and 10 control subjects revealed dysplastic changes in eight autistic (67%) and two (20%) control subjects. Focal disorganization of the cytoarchitecture, deficit and altered morphology and spatial orientation of Purkinje, granule, basket, stellate and unipolar brush cells are indicators of profound disruption of flocculus circuitry. In the flocculus of the autistic subjects, the volume of Purkinje cells was 30% less in the dysplastic than in the not-affected area ($p < 0.01$). Moreover, in the entire cerebellum of the autistic subjects the volume of Purkinje cells was 25% less than in the control subjects ($p < 0.001$). These data suggest that floccular dysplasia plays a pivotal role in dysfunction of the oculomotor system in autism.

4. Clinicopathological Stratification of Idiopathic Autism and Autism Associated with Duplications 15q11.2-q13

Wegiel J, Schanen NC, Cook EH, Brown WT, Kuchna I, Nowicki K, Wegiel J, Imaki H, Ma SY, London E, Wisniewski T. Chapter in press: The Neuroscience of Autism Spectrum Disorders; Editors: Joseph Buxbaum and Patrick Hof, Elsevier Inc. 2013

See Proj 1 Report (Dr. Thomas Wisniewski)

5. Brain region– and neuron-type–specific delay of neuronal growth desynchronizes brain development and maturation in autism

Wegiel J, Flory M, Kuchna I, Nowicki K, Ma SY, Imaki H, Wegiel J, Cohen IL, London E, Brown WT, Wisniewski T. Original paper. Manuscript submitted to Acta Neuropathologica

The prevalence of research focused on the cortex results in a mainly corticocentric theory of autism (Frith 2004, Geschwind and Levitt 2007). However, all three diagnostic modalities of autism engage subcortical structures including (a) the amygdala, in processing social information and involved in emotional interpretation, fear and anxiety (Amaral et al 2003, Baron-Cohen et al 2000, Winston et al 2002); (b) the thalamus, involved in language functions, attention, anxiety and obsessive thinking (Ojemann 1971,1977, Oke et al 1978); (c) the striatum, linked to repetitive motor behaviors, compulsions and rituals (Day and Carelli 2007, Salamone 1944, Sears et al 1999); and (d) the brainstem and cerebellar deep nuclei, integrating a cerebellar role in motor functions, language and cognition, and eye motion control (Leyung et al 2000, Sato and Kawasaki 1991). Cholinergic neurons in the nucleus basalis of Meynert control the cortical mantle and play a modulatory role in anxiety, arousal and emotional and motor responses (Kilgard 2003, Murray and Fibiger 1985), whereas the substantia nigra's dopaminergic neurons modulate striatal functions including repetitive behaviors.

The aim of this study was to test the hypothesis that subcortical structures are affected by developmental alterations and contribute in parallel with cortical networks to global brain developmental defects of connectivity and resulting functional deficits in autism. The study expands corticocentric theory with evidence that autism is associated with delayed and desynchronized neuron growth in early childhood in both cortical and subcortical gray matter and accelerated but still desynchronized neuron growth in late childhood and adulthood. The volume of the neuronal soma and the nucleus was estimated in 16 brain structures and their 19 cytoarchitectonic subdivisions in 13 autistic and 14 control subjects with ages ranging from 4 to 64 years. A significant deficit of neuron soma volume ($p < 0.001$) was detected in 89% of the structures examined, including all 16 brain structures and 15 of 19 of their anatomical subdivisions in 4-8 year old children with autism. A very severe volume deficit in 17%, severe in 44%, moderate in 22% and mild in 17% of brain structures of autistic subjects is a sign of desynchronized development of anatomically and functionally related neurons that may explain social and communication deficits, and restricted repetitive and stereotyped patterns of behavior. Reduction of the developmental deficit from on average 19.6% in 4-8 year old to 8.8% in >8 year old subjects, indicates delayed acceleration of growth of neurons in late childhood and adulthood. Brain region and neuron-type specific volume deficits reflect desynchronized neuron and neuronal networks growth. The most severe delay in 4-8 year old autistic children suggests that deregulation of brain development before the 4th year defines autism encephalopathy and dysfunction for life.

6. Delayed development of claustrum in autism

Wegiel J, Morys J, Ma SY, Kuchna I, Nowicki K, Imaki H, Wegiel J, Flory M, Brown WT, Wisniewski T. Chapter in preparation for submission: Functional Neuroanatomy of the Claustrum. Edited by: John Smythies, Lawrence Edelstein, V.S. Ramachandran, Elsevier 2013

The claustrum receives inputs from many cortical areas, integrates multiple inputs into a new signal and redirects sensory information throughout the striatum and thalamus. Interconnectivity with subcortical nuclei and sensory cortical areas indicates the claustrum's involvement in sensorimotor integration and potentially the most complex human brain function —consciousness, as well as in higher orders of functionality enabling the organism to rapidly adapt to the subtleties and nuances of a changing environment (Edelstein and Denaro 2004). The attraction to routines and sameness appears to be one of the very striking behavioral alterations characteristic of autism. It appears that claustrum immaturity, reflected in the neuronal soma deficit of 29 % in children and of 17 % in adults, and the very striking deficit of neuronal nucleus volume (42 % and 22 %, respectively), may be responsible for the claustrum neurons' functional impairment and deficits of adaptability and consciousness. The mean volume of the neuronal body in 4 to 8-year old autistic children was less by 29% (1,410 μm^3, n = 4) than in control group (1,999 μm^3; n = 4; $p < 0.000$). However, the mean volume of neuronal body in 13 to 36-year-old autistic subjects was less only by 17% (1,582 μm^3; n = 5) than in control group (1,904 μm^3; n = 5; $p < 0.000$). The mean volume of neuron nucleus in 4 to 8-year old autistic children (234 μm^3; n = 4), was 42% less than in the control group (400 μm^3; n = 4; $p < 0.000$). The mean volume of the neuronal nucleus in 13 to 36-year-old autistic subjects was 22% less (266 μm^3) than in the control group (342 μm^3). Very severe volume deficit in 4 to 8-year old autistic subjects and reduction of this deficit by 38% in cell soma volume and by 48% in the volume of nucleus volume in subjects more than 8 year old suggests delay of neuron growth in younger group and abnormal acceleration in late childhood. These data suggest also that inhibition of neuron growth is the result of altered regulation of neuron growth before age of 4 years and results in lifelong structural and functional abnormalities. Altered trajectory of neuron growth in the claustrum in comparison to other brain structures reflects both local and global dysregulation of claustrum and claustrum connectivity.

7. Different trajectories of abnormal neuronal growth desynchronize brain development in autism associated with dup15 and autism of unknown origin

Wegiel J, Flory M, Kuchna I, Nowicki K, Ma SY, Imaki H, Wegiel J, Cohen IL, London E, Brown WT, Wisniewski T. Original study. Project in progress

The comparison of neuron growth in dup(15) autism and in idiopathic autism reveals such common features as: 1. Developmental neuronal volume deficit, 2. Brain region specific deficits that reflect desynchronized brain development. 3. Multiregional alterations that indicate global developmental encephalopathy.

Differences between idiopathic autism and dup15 associated autism:

1. Different region specific neuron volume deficit.
2. Different trajectory of neuron growth with severe developmental delay in early childhood and significant correction in late childhood in idiopathic autism, and smaller but permanent neuron growth arrest in autism associated with dup(15).

8. Amyloid-beta and lipid oxidation in the brain cortex in autism
Frackowiak J, Mazur-Kolecka B, Kuchna I, Brown WT, Wegiel J.
Poster presented at the Cell Development meeting at Santa Cruz, August 2012.
Original study, project in progress.

In children with severe autism and aggression plasma levels of secreted beta amyloid precursor protein (APP) are two times higher than in children without autism and up to 4 times higher than in children with mild autism (Sokol et al 2006, Ray et al 2011). Increased levels of secreted amyloid precursor protein alpha (sAPP-α) were increased in 60% of autistic children, as compared to age-matched controls (Bailey et al 2008). Ray et al (2011) hypothesized that increased processing of APP by alpha-secretases contributes to autism. Immunocytochemical studies of the brain of autistic subjects revealed enhanced accumulation of amino-terminally truncated Aβ in cortex, subcortical structures and cerebellum (Wegiel et al 2012). Pathological effects of N-terminally truncated Aβ are not known but contribution of oxidative stress to Aβ accumulation has been suggested. In autistic subjects accumulation of intracellular Aβ in neurons correlates with colocalization with lipid peroxidation products, 4-hydroxy-2-nonenal (HNE) and malodialdehyde (MDA). It suggests that intracellular Aβ is the source of oxidative stress rather than product of oxidative stress. Therefore enhanced APP processing and intracellular Aβ accumulation might be the trigger of enhanced oxidative stress and accumulation of lipids peroxidation products with functional consequences.

KEY RESEARCH ACCOMPLISHMENTS

Project 2 integrates three major research strategies including the study of the contribution of:

1. Qualitative developmental abnormalities to the autistic phenotype.
2. Quantitative developmental abnormalities to the autistic phenotype.
3. Developmental neuronal metabolic alterations to the clinical phenotype of autism

Major accomplishments:

1. Thanks to DOD grant and Autism Speaks and Autism Tissue Program support in tissue acquisition in past four years, we were able to preserve historically the largest collection of unique quality brain tissue samples (72 brain hemispheres) including:
   - 32 brain hemispheres of people with idiopathic autism,
   - 12 brains hemispheres of people with dup15 autism,
   - 28 control brain hemispheres.
2. Brain hemispheres cut into serial sections provided material for several research strategies. Therefore we were able to expand spectrum of research targets and hypotheses tested in this Program Project.
3. Neuropathological component (Project #1) provided neuropathological reports and exclusion criteria reducing risk of distortion of research results by comorbidity, pre-, peri- and postmortem changes.
4. The study determined the contribution of qualitative developmental abnormalities to the autistic phenotype in autism with an unknown etiology and autism caused by maternal origin dup(15) (Wegiel et al 2010a and b Wegiel et al 2012).

5. We identified both, (a) differences between the pattern of developmental abnormalities in autism associated with duplications 15q11.2-q13 and autism of unknown origin and (b) core neuropathology present in autism regardless of autism etiology.
6. Project results in detection of focal abnormalities thatplay a key role in early onset of epilepsy, functional regression and an increased risk of Sudden Unexpected Death in Epilepsy (SUDEP).
7. This study integrates (i) localized models of defective development of neurons with (ii) more complex models of altered neuronal circuits into (iii) a global model of brain development desynchronization.
8. Unbiased stereology of 16 brain structures and 19 anatomical subdivisions identified desynchronization of development of neurons, neuronal circuits and neurotransmitter systems as the major contributor to the autistic phenotype.
9. Mapping of these abnormalities to structures with their known role in social behavior, communication, and stereotypic behavior results in identification of a structural component of functional deficits observed in clinical studies.
10. Enhanced $A\beta_{17\text{-}40/42}$ immunoreactivity observed in neurons in more than 50% of subjects diagnosed with idiopathic autism, and a more pronounced Aβ load in the majority of individuals diagnosed with dup15 and autism, including children, suggests an early and significant alteration of APP processing with α-secretase.
11. The presence of $A\beta_{1\text{-}40/42}$ in diffuse plaques in three autistic subjects, 39 to 52 years of age, suggests there is an age-associated risk of metabolic developmental alterations with an intraneuronal accumulation of a short form of Aβ and an extracellular deposition of full length Aβ in nonfibrillar plaques.

REPORTABLE OUTCOMES

1. Wegiel J, Wisniewski T, Chauhan A, Chauhan V, Kuchna I, Nowicki K, Imaki H, Wegiel J, Ma SY, Wierzba-Bobrowicz T, Cohen IL, London E, Brown WT. Type, topography and sequelae of neuropathological changes shaping clinical phenotype of autism. In: Autism: Oxidative Stress, Inflammation, and Immune Abnormalities. Ed.: Abha Chauhan, Ved Chauhan and W. Ted Brown. Taylor & Francis/CRC Press, Boca Raton, FL, 2010, pp. 1-34.

2. Wegiel J, Kuchna I, Nowicki K, Imaki H, Wegiel J, Marchi E, Ma SY, Chauhan A, Chauhan V, Wierzba Bobrowicz T, de Leon M, Saint Louis LA, Cohen IL, London E, Brown WT, Wisniewski T. The neuropathology of autism: defects of neurogenesis and neuronal migration, and dysplastic changes. Acta Neuropathologica, 2010, 119,755-770.

3. Wegiel J, Schanen CN, Cook EH, Sigman M, Brown WT, Kuchna I, Nowicki K, Wegiel J, Imaki H, Ma SY, Marchi E, Wierzba-Bobrowicz T, Chauhan A, Chauhan V, Cohen IL, London E, Flory M, Lach B, Wisniewski T. Differences Between the Pattern of Developmental Abnormalities in Autism Associated with Duplications 15q11.2-q13 and Idiopathic Autism. J Neuropathol Exp Neurol 2012, 71, 382-397.

4. Wegiel J, Frackowiak J, Mazur Kolecka B, Schanen NC, Cook EH, Sigman M, Brown WT, Kuchna I, Wegiel J, Nowicki K, Imaki H, Ma SY, Chauhan A, Chauhan V, Miller DL,

Mehta PD, Cohen IL, London E, Reisberg B, de Leon MJ, Wisniewski T. Amyloid Abnormal Intracellular Accumulation and Extracellular Aβ Deposition in Idiopathic and Dup15q11.2-q13 Autism Spectrum Disorders. PloS ONE 2012, 7, e35414.

5. Wegiel J, Kuchna I, Nowicki K, Imaki H, Wegiel J, Ma SY, Azmitia EC, Banerjee P, Chauhan A, Chauhan W, Cohen IL, London E, Brown WT, Wisniewski T. Contribution of Olivo-floccular Circuitry Developmental Defects to Atypical Gaze in Autism. Submitted to J Autism and Dev Disabilities.

6. Wegiel J, Schanen NC, Cook EH, Brown WT, Kuchna I, Nowicki K, Wegiel J, Imaki H, Ma SY, London E, Wisniewski T. Clinicopathological Stratification of Idiopathic Autism and Autism Associated with Duplications 15q11.2-q13. Chapter in press: The Neuroscience of Autism Spectrum Disorders; Editors: Joseph Buxbaum and Patrick Hof, Elsevier Inc. 2013

7. Wegiel J, Flory M, Kuchna I, Nowicki K, Ma SY, Imaki H, Wegiel J, Cohen IL, London E, Brown WT, Wisniewski T. Brain region– and neuron-type–specific delay of neuronal growth desynchronizes brain development and maturation in autism. Submitted to Acta Neuropathologica.

8. Wegiel J, Morys J, Ma SY, Kuchna I, Nowicki K, Imaki H, Wegiel J, Flory M, Brown WT, Wisniewski T. Delayed development of claustrum in autism. Chapter in preparation for submission: Functional Neuroanatomy of the Claustrum. Edited by: John Smythies, Lawrence Edelstein, V.S. Ramachandran, Elsevier 2013

9. Wegiel J, Flory M, Kuchna I, Nowicki K, Ma SM, Imaki H, Wegiel J, Cohen IL, London E, Brown WT, Wisniewski T. Different trajectories of abnormal neuronal growth desynchronize brain development in autism associated with dup15 and autism of unknown origin. Project in progress.

10. Frackowiak J, Mazur-Kolecka B, Kuchna I, Brown WT, Wegiel J. Amyloid-beta and lipid oxidation in the brain cortex in autism. Project in progress. Poster presented at the Cell Development meeting at Santa Cruz, August 2012. Paper in preparation for submission.

11. Wegiel J, Lightfoot D, Pickett J, Brown WT. New trends in brain tissue banking for autism research. Autism Spectrum News 2012, 4, no3.

Meetings (2012)

1. Wegiel J, Schanen NC, Cook EH, Brown WT, Kuchna I, Nowicki K, Wegiel J, Imaki H, Ma SY, London E, Wisniewski T. Results of application of new methods of tissue handling, distribution, and sharing for research on autism. Contributions of postmortem tissue to the study of Developmental Disorders. 20th Anniversary of the NICHD Brain and Tissue Bank for Developmental Disorders. Bethesda, July 16-17, 2012

2. Wegiel J, Schanen NC, Cook EH, Brown WT, Kuchna I, Nowicki K, Wegiel J, Imaki H, Ma SY, London E, Wisniewski T. Clinicopathological stratification of idiopathic autism and

autism associated with duplications 15q11.2-q13. Dup15q Alliance 2012 Scientific Meeting. Boston Children's Hospital, Boston August 9-10, 2012

3. Frackowiak J, Mazur-Kolecka B, Kuchna I, Brown WT, Wegiel J. Amyloid-beta and lipid oxidation in the brain cortex in autism. Cell Development Meeting at Santa Cruz, August 2012.

CONCLUSIONS

Delayed, desynchronized growth of neurons in all examined cortical and subcortical structures is the major marker of developmental defects contributing to autism phenotype

1. Deficit of neuron volume detected in all 16 brain structures and 15 of 19 of their anatomical subdivisions in 4-8 year old children with autism is an indicator of global developmental encephalopathy in autism of unknown origin.
2. A very severe volume deficit in 17%, severe in 44%, moderate in 22% and mild in 17% of brain structures of autistic subjects is a sign of desynchronized development of anatomically and functionally related neurons that may explain social and communication deficits, and restricted repetitive and stereotyped patterns of behavior.
3. Reduction of the developmental deficit from on average 19.6% in 4-8 year old to 8.8% in >8 year old subjects, indicates delayed acceleration of growth of neurons in late childhood and adulthood.
4. The most severe delay in 4-8 year old autistic children suggests that deregulation of brain development before the 4th year defines autism encephalopathy and dysfunction for life.
5. The study expands corticocentric theory with evidence that autism is associated with delayed and desynchronized neuron growth in early childhood in both cortical and subcortical gray matter and accelerated but still desynchronized neuron growth in late childhood and adulthood.

Focal dysplasia and heterotopias are the major cause of increased risk of sudden unexpected death in early childhood in autism associated with dup15

1. Severe microcephaly, with brain weight reduced by 300 g is one of the most significant signs of global encephalopathy increasing the risk of epilepsy.
2. 2.8 times more frequent developmental alterations, especially common in the hippocamapl formation of autistic subjects with dup15, and presence up to 11 different types of developmental alterations are the major contributor to early onset of epilepsy and high risk of SUDEP.
2. Reduced volume of neurons in a majority of subcortical structures and some cortical regions in the brain of autistic children 4–8 years of age appear to reflect brain immaturity in early childhood contributing to autism and intellectual deficit.
3. Combination of all of these developmental defects increases risk of death at a very early age (~10 years) in autism associated with dup15.

Metabolic alterations reflected in enhanced accumulation of truncated Aβ and oxidative stress contribute to autistic phenotype

1. Abnormal accumulation of amino-terminally truncated Aβ in neurons and glial cells is a common finding in the brain of autistic children and young adults.
2. Enhanced accumulation is brain region and cell type specific.
3. These developmental alterations are more severe in brains of idic15 subjects diagnosed with autism and epilepsy than in idiopathic autism.
4. The presence of Aβ in lysosomes, autophagic vacuoles and lipofuscin, as well as presence of Aβ not associated with these structure, suggests that enhanced intracellular accumulation of Aβ in autism result from different pathways of APP processing and Aβ deposition.
5. Detection of increased levels of Aβ complexes in the soluble and insoluble form in brain cortex and cerebellum of autistic subjects, indicates brain structure-specific alteration of APP processing and Aβ trafficking in autism.
6. Abnormalities of Aβ intracellular accumulation in early stage of brain development suggest their link to the clinical phenotype, including seizures, in idiopathic autism and autism associated with idic15.
7. Aβ accumulation in neurons initiates oxidative stress resulting in lipids peroxidation. Accumulation of Aβ in activated astrocytes results in their death. Both are markers of developmental alterations in APP processing with structural and probably functional consequences.

The link between developmental alterations in the oculomotor system and abnormal gaze in autism

1. The flocculus and the inferior olive are the components of the olivo-cerebellar system involved in control of oculomotor function and gaze control. The study revealed that the flocculus is affected by dysplastic changes in 67% of autistic subjects.
2. Disorganization of the granule, molecular and Purkinje cell layer, striking deficit of Purkinje cells, their abnormal spatial orientation, severe deficit and distortion of the Purkinje cells' dendritic tree are the major structural defects in the dysplastic portion of the flocculus.
3. The volume of Purkinje cells in the flocculus of control subjects is 20% less (8,865 um^3) than in other parts of the cerebellar cortex (11,092 um^3, $p<0.03$). In autistic subjects the volume of Purkinje cells is significantly less than in control subjects in the entire cerebellar cortical ribbon ($p<0.001$).
4. Severe developmental abnormalities in the flocculus combined with reduced volume of Purkinje cells in the entire cerebellum of autistic subjects but no changes in morphology and neuronal size in the inferior olive, the second component of the olivo-floccular integrator of oculomotor function, suggests that mechanism leading to floccular dysplasia may play a pivotal role in defective function of the oculomotor system in autism.

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APPENDICES

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ORIGINAL ARTICLE

Differences Between the Pattern of Developmental Abnormalities in Autism Associated With Duplications 15q11.2-q13 and Idiopathic Autism

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Abstract

The purposes of this study were to identify differences in patterns of developmental abnormalities between the brains of individuals with autism of unknown etiology and those of individuals with duplications of chromosome 15q11.2-q13 (dup[15]) and autism and to identify alterations that may contribute to seizures and sudden death in the latter. Brains of 9 subjects with dup(15), 10 with idiopathic autism, and 7controls were examined. In the dup(15) cohort, 7 subjects (78%) had autism, 7 (78%) had seizures, and 6 (67%) had experienced sudden unexplained death. Subjects with dup(15) autism were microcephalic, with mean brain weights 300 g less (1,177 g) than those of subjects with idiopathic autism (1,477 g; $p < 0.001$). Heterotopias in the alveus, CA4, and dentate gyrus and dysplasia in the dentate gyrus were detected in 89% of dup(15) autism cases but in only 10% of idiopathic autism cases ($p < 0.001$). By contrast, cerebral cortex dysplasia was detected in 50% of subjects with idiopathic autism and in no dup(15) autism cases ($p < 0.04$). The different spectrum and higher prevalence of developmental neuropathologic findings in the dup(15) cohort than in cases with idiopathic autism may contribute to the high risk of early onset of seizures and sudden death.

Key Words: Autism, Chromosome 15q11.2-q13 duplication, Developmental brain alterations, Seizures, Sudden unexpected death.

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INTRODUCTION

Autism is the most severe form of autism spectrum disorder (ASD) and is characterized by qualitative impairments in reciprocal social interactions, qualitative impairments in verbal and nonverbal communication, restricted repetitive and stereotyped patterns of behavior, interests and activities, and onset before the age of 3 years (1). Autism is heterogeneous, both phenotypically and etiologically. In 44.6% of affected children, autism is associated with cognitive impairment as defined by intelligence quotient scores of less than 70 (2). Epilepsy is a comorbid complication diagnosed in up to 30% of individuals with autism (3). In 90% to 95% of cases, the etiology of autism is not known (idiopathic or nonsyndromic autism) (4, 5). Twin and family studies have indicated both a strong and a moderate heritability for ASD (6–8).

Approximately 5% to 10% of individuals with an ASD have an identifiable genetic etiology corresponding to a known single gene disorder, for example, fragile X syndrome or chromosomal rearrangements, including maternal duplication of 15q11-q13. Supernumerary isodicentric chromosome 15 [idic(15)] (formerly designated as inverted duplication 15)

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is a relatively common genetic anomaly that most often leads to tetrasomy or mixed trisomy/tetrasomy of the involved segments and arises from a U-type crossover between a series of low copy repeats (LCRs) located on the proximal long arm. Small heterochromatic idic(15) chromosomes, which do not include the 15q11-q13 region, are often familial and are not associated with a clinical abnormality (9, 10). The symptoms in people with idic(15) markers correlate with the extent of duplication of the Prader-Willi syndrome/Angelman syndrome critical region (15q11-q13) (10, 11). Larger supernumerary idic(15) chromosomes, which include the imprinted chromosome 15q11-q13 region, are associated with a cluster of clinical features that include intellectual deficits (IDs), seizures, autistic behavior, hypotonia, hyperactivity, and irritability (12). Duplications of chromosome 15q11-q13 account for approximately 0.5% to 3% of ASD and may be the most prevalent cytogenetic aberration associated with autism in most studies. These duplications range from 4 to 12 Mb and may occur either through generation of supernumerary idic(15) chromosomes or as interstitial duplications and triplications. For interstitial duplications, maternal origin confers a higher risk for an abnormal phenotype (13, 14), and most of the reported chromosome 15 duplications (dup[15]) are maternally derived. A small number of subjects with duplications of paternal origin have been variously reported as being unaffected (13, 15–17), affected but without ASD (16, 18), or affected with ASD (19). Interstitial triplications (int trp[15]) are relatively rare but have invariably been associated with a severe phenotype, including ID, ASD, or autistic features, and frequently with seizures. The parent-of-origin effect is not evident in the reported cases of int trp(15), with both maternal and paternal triplications associated with poor outcome.

Clinical studies indicate that most individuals diagnosed with dup(15) of maternal origin fulfill the criteria for the diagnosis of autism. In the first clinical reports, 24 individuals with idic(15) and autism were identified using standardized criteria of the *Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised* (13, 15, 20–24). In several other studies of subjects with idic(15) chromosomes, autism was clinically diagnosed, although without the application of standardized measures of autism (25–27). Application of the Gilliam Autism Rating Scale (28) to another idic(15) cohort confirmed a high prevalence of autism; 20 (69%) of 29 children and young adults with idic(15) had an ASD (29).

TABLE 1. Material Examined

Group	Case No.	Sex	Age, y	Cause of Death	PMI, h	Hem	Brain Weight, g
dup(15) autism	1	M	9	SUDEP	13.6	R	1,130
	2	M	10	SUDEP	17.7	R	1,070
	3	M	11	SUDEP	10.5	R	1,540
	4	F	15	SUDEP (suspected)	24.0	LR	1,141
	5	F	15	Pneumonia	—	LR	1,125
	6	M	20	Cardiopulmonary arrest (choking on food)	28.1	L	1,190
	7	M	24	Pneumonia	36.3	L	1,200
	8	F	26	SUDEP (suspected)	28.6	R	1,310
	9	F	39	SUDEP	32.8	R	890
			Mean (SD)		23.9 (9.2)		1,177 (177)
Idiopathic autism	1	M	2	Asphyxia (drowning)	4.0	R	1,328
	2	F	5	Asphyxia (drowning)	33.0	R	1,360
	3	M	5	Asphyxia (drowning)	25.5	R	1,560
	4	M	8	Asthma attack	13.8	R	1,740
	5	M	9	SUDC	3.7	R	1,690
	6	M	11	Asphyxia (drowning)	—	R	1,400
	7	M	28	Seizure-related	43.0	R	1,580
	8	M	32	Brain tumor (glioblastoma multiforme)	—	L	1,260
	9	M	51	Heart failure	22.2	R	1,530
	10	M	52	Heart failure	11.5	L	1,324
			Mean (SD)		19.6 (14.0)		1,477 (166)
Control	1	F	8	Rejection of cardiac transplant	20.0	R	1,340
	2	M	14	Asphyxia (hanging)	5.0	R	1,420
	3	M	14	Multiple traumatic injuries	16.0	R	1,340
	4	M	32	Heart failure	14.0	R	1,401
	5	F	33	Bronchopneumonia	6.0	L	1,260
	6	F	43	Sepsis	10.1	L	1,350
	7	M	47	Myocardial infarct	23.0	L	1,450
			Mean (SD)		13.4 (6.8)		1,366 (63)

PMI, postmortem interval; Hem, hemisphere; R, right; L, left; SUDEP, sudden unexpected death of subject with known epilepsy: SUDC, sudden unexpected death in childhood.

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TABLE 2. Chromosome 15 Abnormalities in the dup(15) Cohort

Case #	Chromosomal Alterations	Prader-Willi Syndrome/Angelman Syndrome Critical Region (PWS/ASCR)	Parental Origin of Abnormality
1	47,XY,+idic(15)(q13;q13); Idic(15) arising from BP3:BP3 exchange. Subject 02-18, Wang et al (47)	Tetrasomy	Maternal
2	47,XY,+idic(15)(q13;q13); Idic(15) arising from BP4:BP5 exchange. Subject 01-22, Wang et al (47)	Tetrasomy	Maternal
3	47,XY,+der(15)(pter>q13::q13>cen>q13::q13>pter). Tricentric chromosome 15 arising from BP3:BP3 exchanges. Case 2, Mann et al (46); Subject 00-29, Wang et al (47)	Hexasomy	Maternal
4	47,XX,+idic(15)(q13;q13); Idic(15) arising from BP4:BP5 exchange. Subject 99-93, Wang et al (47)	Tetrasomy	Maternal
5	47,XX,+der(15)(q13;q13); Idic(15) arising from BP4:BP5 exchange	Tetrasomy	Maternal
6	IDIC15 (2 extra copies of region from beginning of array to BP4)	Tetrasomy	Not determined
7	47,XY,del(15)(q11.2)+idic(15)(q13;q13); Idic(15) arising from BP4:BP5 exchange; deletion of BP1:BP2 on 1 homolog of chromosome 15. Subject 00-03, Wang et al (47)	Tetrasomy	Maternal
8	47,XX,+idic(15)(q13;q13); Idic(15) arising from BP4:BP5 exchange. Subject 99-27, Wang et al (47)	Tetrasomy	Maternal
9	46,XX,trp(15)(q11.2q13). Subject 02-9, Wang et al (47)	Tetrasomy	Maternal

PWS/ASCR, Prader-Willi syndrome/Angelman syndrome critical region.

The link between the extent of genetic duplication and clinical phenotype has not yet been determined, but genomic and functional profiling provides insights into the direct and indirect effects of the copy number gains associated with chromosome 15 duplications. The γ-aminobutyric acid type A ($GABA_A$) receptor subunit genes ($\alpha 5$, β, and $\gamma 3$) that have been implicated in the etiology of autism (30) are located in the susceptibility segment of duplicated chromosome 15

TABLE 3. Autism Diagnostic Interview-Revised–Based Diagnosis of Autism

Group	Case No.	Reciprocal Social Interactions (10)	Communication: Verbal (8)	Communication: Nonverbal (7)	Restricted, Repetitive, and Stereotyped Behavior (3)	Alterations Evident Before 36 mo (1)	Diagnosis (Test)
dup(15) autism	1	7	NA	0	0	5	Autism (ADOS-G)
	2	24	NA	11	5	3	Autism (ADI-R)
	3	18	NA	2	2	5	PDD-NOS
	4	26	22	NA	12	5	Autism (ADI-R)
	5	—	—	—	—	—	Autism (ADI-R) (score not available)
	6	27	18	NA	4	5	Autism (ADI-R)
	7	23	22	NA	9	3	Autism (ADI-R)
	8	28	16	NA	9	5	Autism (ADI-R)
	9						Unknown
Idiopathic autism	1	14	NA	9	6	5	Autism (ADI-R)
	2						Autism (ADOS)
	3	22	NA	14	6	5	Autism (ADI-R)
	4	11	NA	8	2	4	Atypical autism – ASD (ADI-R)
	5	26	NA	12	5	4	Autism (ADI-R)
	6	25	20	NA	4	4	Autism (ADI-R)
	7	22	16	NA	5	3	Autism (ADI-R)
	8	—	—	—	—	—	Autism (ADI-R) (score not available)
	9	27	19	9	6	5	Autism (ADI-R)
	10	—	—	—	—	—	Atypical autism – ASD (ADI-R score not available)

ADI-R, Autism Diagnostic Interview – Revised (cutoff scores); ADOS-G, Autism Diagnostic Observation Scale – Generic (42); NA, not applicable; PDD-NOS, Pervasive Developmental Disorder – Not Otherwise Specified.

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TABLE 4. Behavioral, Neurologic, and Other Clinical Observations

Group	Case No.	Psychiatric Disorders and Neurologic Symptoms	Cognitive Assessment	Seizures (Age at Onset)
dup(15) autism	1	Severe hypotonia. Regression in infancy. Abnormal response to pain and heat.	Profound ID (DQ < 20)	Infantile spasms. Intractable epilepsy (10 mo)
	2	Hypotonia. Severe regression at age of 15 mo. Head banging.	Profound ID (DQ = 22)	Intractable epilepsy (8 mo)
	3	Regression with infantile spasms. Severe hypotonia.	Profound ID (DQ < 20)	Infantile spasms. Intractable epilepsy (10 mo). Vagus nerve stimulator.
	4	Delay of motor skills. Mild to moderate spastic quadriparesis. Abnormal response to pain, cold.	Severe ID (DQ = 31)	Seizures (11 y)
	5	Hyperactive, verbal	(—)	No record
	6	Sleep disorder. Abnormal response to pain, heat and cold.	(—)	No record
	7	Abnormal gait	Profound ID (DQ < 20)	Intractable epilepsy (7 y). Vagus nerve stimulator. Callosotomy.
	8	Obsessive compulsive symptoms	Moderate ID (IQ = 36)	Epilepsy (16 y)
	9	Cerebral palsy. Microcephaly	Severe ID.	Intractable epilepsy (9 y). Vagus nerve stimulator.
		Aggressive behavior. Trichotillomania.	(—)	
Idiopathic autism	1	Self-stimulatory behavior	(—)	No record
	2	Hyperactivity, attention deficit. Sleep disorder. Enhanced sensitivity to light and sound.	Cognitive delay (IQ = 65)	No record
	3	Sleep disorder	(—)	No record
	4	Self-stimulatory behavior	(—)	Epilepsy (8 y)
	5	Hypotonia	Moderate ID	No record
	6	No record	Moderate ID	Epilepsy
	7	Bipolar disorder	(—)	Epilepsy
	8	No record	(—)	No record
	9	Enhanced sensitivity to sound, heat, and cold. Low pain threshold.	(—)	One grand malseizure
	10	Bipolar disorder, social anxiety	(—)	No record

DQ, developmental quotients; ID, intellectual deficit; —, no formal assessment of ID available.

(31–34). A map of parent-of-origin-specific epigenetic modifications suggests that this imprinted locus may have links not only with autism but also with other psychiatric phenotypes (35). Differential methylations in the 15q11-q13 region, including the $GABA_A$ gene (30, 36–38), may contribute to epigenetic modifications and a broader clinical phenotypes in dup(15)/autism. Several other genes located in or near the 15q11-q13 region may contribute to a variable phenotype of autism, including a gene for juvenile epilepsy located near D15S165 (39) and a locus for agenesis of the corpus callosum (40).

We hypothesized that the neuropathology of autism with dup(15) differs from that of idiopathic autism and that it would provide an explanation for the high prevalence of seizures and associated sudden death in the dup(15) cohort. The aim of this comparative postmortem study of the brains of individuals diagnosed with idic(15) or int trp(15) (collectively referred to as dup[15]) and of individuals with idiopathic autism was to identify common neuropathologic developmental defects for both cohorts and the patterns of changes distinguishing dup(15) from idiopathic autism. The dup(15) cohort examined exhibited a strikingly high prevalence of epilepsy, including intractable epilepsy, and a high rate of sudden unexpected death in childhood and early adulthood. Therefore, the second aim of the study was to identify patterns of neuropathologic changes that may contribute to epilepsy and sudden death in the dup(15) cohort.

MATERIALS AND METHODS

The cohort of subjects diagnosed with dup(15) consisted of 9 subjects (range, 9–39 years), including 5 males (55%) and 4 females (45%). The cohort with autism consisted of 10 subjects (range, 2–52 years), including 9 males (90%) and 1 female (10%). The control cohort consisted of 7 subjects from 8 to 47 years, including 4 males (43%) and 3 females (57%). The brain of 1 subject diagnosed with dup(15) was excluded because of very severe autolytic changes, and the brain of 1 control subject was excluded because of lack of information about cause of death. The mean postmortem interval varied from 23.9 hours in the dup(15) cohort, to 19.6 hours in the idiopathic autism cohort, and 13.4 hours in the control group (Table 1).

Clinical and Genetic Characteristics

Psychological, behavioral, neurologic, and psychiatric evaluation reports and reports by medical examiners and pathologists were the source of the medical records of the examined postmortem subjects. Medical records were obtained after consent for release of information from the subjects' parents. In

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TABLE 5. Topography and Type of Major Developmental Alterations

		Hippocampus		Cerebral cortex	Cerebellum	
Group	**Case No.**	**Heterotopia (Alveus, CA4, DG)**	**Dysplasia (DG)**	**Dysplasia**	**Heterotopia**	**Dysplasia**
dup(15) autism	1	+	++		+	
	2	+	++			+
	3	+	++		+	+
	4	+				+
	5	+	+++		++	
	6	+	++			+
	7	+	+++++		++	+
	8		+++		+	+
	9	++	+++			—
	8 (89%)	8 (89%)	0	5 (56%)	6/8 (75%)	
Idiopathic autism	1		+	+	+	+
	2			+		+
	3			+		+
	4	+		+	+	+
	5				+	
	6				+	—
	7			+	+	
	8				+	
	9					—
	10					
	1 (10%)	1 (10%)	5 (50%)	6 (60%)	4/8 (50%)	
Control	1					
	2					
	3					
	4					
	5					
	6					
	7					+
		0	0	0	0	1 (14%)
p						
dup(15) vs idiopathic autism		0.001	0.001	0.03	ns	ns
dup(15) vs control		0.001	0.001	ns	0.03	0.04
Autism vs control		ns	ns	0.04	0.03	ns

+, The number of types of developmental alterations and percentages of subjects with developmental defects are in parentheses; ns, not significant; —, missing structure; DG, dentate gyrus.

Statistical analyses: Fisher exact test and Mann-Whitney *U* test.

most subjects diagnosed as being affected by dup(15) and idiopathic autism, the Autism Diagnostic Interview – Revised (ADI-R) was administered to the donor family after the subject's death as a standardized assessment tool to confirm an autism diagnosis (41). In addition, 6 of the subjects with dup(15) chromosomes were enrolled in a study of molecular contributors to the phenotype. The study was approved by the institutional review boards of the University of California, Los Angeles, and Nemours Biomedical Research. Before their deaths, 6 subjects (Cases 1–4, 7, and 8) had undergone behavioral and cognitive testing using the ADI-R, the Autism Diagnostic Observation Scale – Generic (ADOS-G) (42), and the Mullen Scales of Early Learning (43, 44) or the Stanford-Binet Intelligence Scales (45). Age at evaluation ranged from 45 to 251 months.

Molecular genetic evaluation using antemortem peripheral blood samples and lymphoblast cell lines for 8 of the dup(15) cases included genotyping with 19 to 33 short tandem repeat polymorphisms from chromosome 15, Southern blot analysis of dosage with 5 to 12 probes, and measurement of the methylation state at *SNRPN* exon α, as described (46). In addition, array comparative genomic hybridization was performed, using a custom bacterial artificial chromosome array (47). Morphology of the duplication was confirmed by fluorescent in situ hybridization using 5 to 8 probes that detect sequences on chromosome 15q11-q14 (46).

Tissue Preservation for Neuropathologic Study

One brain hemisphere from each subject was fixed in 10% buffered formalin, dehydrated in a graded series of ethanol, infiltrated with polyethylene glycol 400 (no. 807 485; Merck, Whitehouse Station, NJ) and embedded in fresh polyethylene glycol 1000 (48) and stored at 4°C. Tissue blocks were then cut at a temperature of 18°C into 50-μm-thick

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serial sections identified with a number and stored in 70% ethyl alcohol at room temperature. Free-floating hemispheric sections were stained with cresyl violet and mounted with Acrytol. To increase the probability of detection of small focal developmental defects, on average, 140 hemispheric cresyl violet–stained sections at a distance of 1.2 mm were examined per case.

Tissue acquisition for this project was based on individual tissue transfer agreements between the project's principal investigator and several tissue banks, including (i) the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland School of Medicine, Baltimore, MD; (ii) the Harvard Brain Tissue Resource Center, McLean Hospital, Belmont, MA; and (iii) the Brain and Tissue Bank for Developmental Disabilities and Aging of the New York State Institute for Basic Research in Developmental Disabilities. The institutional review board of the Institute for Basic Research in Developmental Disabilities approved the methods applied in this study.

Statistical Analysis

Differences among groups in the presence or absence of heterotopias and dysplasias were examined using the Fisher exact test. Differences in the numbers of abnormalities were examined using the Mann-Whitney *U* (Wilcoxon signed ranks) test or (for comparisons of all 3 groups) the Kruskal-Wallis one-way analysis of variance (an extension of the *U* test), with exact probabilities computed using version 12 of the SPSS statistical package (SPSS for Windows, Release 12.0, 2003; SPSS, Inc, Chicago, IL). Brain weights were compared in one-way analysis of variances controlled for age.

RESULTS

Genetic Characteristics

For 8 subjects (Cases 1–5 and 7–9) in the dup(15) cohort, duplication chromosomes was characterized using a combination of genotyping, fluorescent in situ hybridization, Southern blot, and array comparative genomic hybridization with lymphoblasts generated from antemortem blood samples (Table 2). All were maternally derived; 7 of these subjects were tetrasomic for the imprinted region between brake points (BP)2 and BP3, although the BP involved was variable. The idic(15) present in cells from Case 1 was generated by an exchange between copies of LCR3, causing tetrasomy that extended only to BP3. Four subjects (Cases 2, 4, 5, and 8) had the most common form of idic(15) chromosomes arising by nonallelic homologous recombination (NAHR) between BP4 and BP5, leading to tetrasomy of the region between the p-arm and BP4, with trisomy for the interval between BP4 and BP5. Another subject (Case 7) had a similar idic(15) chromosome but also carried a deletion between BP1 and BP2 on 1 homolog of chromosome 15 (Subject 00-03) (47). Case 3 had a complex tricentric supernumerary chromosome arising from NAHR between multiple copies of BP3, rendering him hexasomic for the region between the centromere and BP3 (46). One subject (Case 9) had an int trp(15) chromosome that led to tetrasomy between BP1 and BP4 and trisomy for the interval between the fourth and fifth LCRs, similar to the dosage seen in the BP4:BP5 idic(15) chromosomes.

The study of 5 subjects with idiopathic autism (Cases 1–4 and 9) revealed the absence of the relevant 15q11-q13 deletion or duplication between BP2 and BP3. In CAL105 normal karyotype was found. In 4 subjects (Cases 5, 7, 8, and 10), frozen tissue and genetic data were not available.

Clinical Characteristics

Of the 9 subjects with dup(15), 7 (78%) were diagnosed with autism (Table 3). In 6 cases, autism was diagnosed clinically and was confirmed with postmortem ADI-R. In a 9-year-old boy (Case 1), autism was diagnosed with ADOS-G (Table 3). This case was reported as Case 2 in Mann et al (46). An 11-year-old boy (Case 3) revealed impairments consistent with the diagnosis of pervasive developmental disorder – not otherwise specified.

In the idiopathic autism group, all 10 subjects were diagnosed clinically as having an ASD. Postmortem ADI-R confirmed a classification of autistic disorder in 6 cases. Two subjects, an 8-year-old boy (Case 4) and a 52-year-old man (Case 10), were diagnosed with atypical autism or high-functioning autism. In a 5-year-old girl (Case 2), autism was diagnosed with ADOS-G. A 32-year-old man (Case 8) and a 52-year-old man (Case 10) were clinically diagnosed as having autism, but the ADI-R could not be conducted postmortem owing to the unavailability of a caregiver who could report on their behavior as a child.

Among the 9 examined subjects with dup(15) chromosomes, 7 individuals were diagnosed with seizures (78%) (Table 4). In 6 cases, death was sudden and unexplained in patients with epilepsy (SUDEP, 6/9 [67%]). In the 10 subjects with autism, epilepsy was diagnosed in 3 (30%) and death was seizure related in 1 (10%; Table 1). In a previously described cohort of 13 subjects with autism who were subject to postmortem examination (49), seizures were reported in 6 (46%) and death was seizure related in 4 (31%).

Brain Weight

The mean brain weight in dup(15) autism was 1,177 g, 300 g less than in idiopathic autism and 189 g less than in the control group (Table 1). Age-adjusted means for these 3 groups were 1,171, 1,474, and 1,378 g, respectively ($F_2 = 9.79$, $p < 0.001$). Post hoc tests showed the difference between the idiopathic and dup(15) groups with autism to be significant (Scheffé-corrected $p = 0.001$). The difference between the dup(15) and control groups was not significant, although suggestive ($p = 0.06$). The difference between the idiopathic and control groups was not significant.

Developmental Abnormalities in Autism Associated With dup(15) and Idiopathic Autism

Three major types of developmental changes, including (i) heterotopias, (ii) dysplastic changes, and (iii) defects of proliferation resulting in subependymal nodular dysplasia, were detected in both the dup(15) (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A323) and idiopathic autism (Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A324) cohorts. In the control group,

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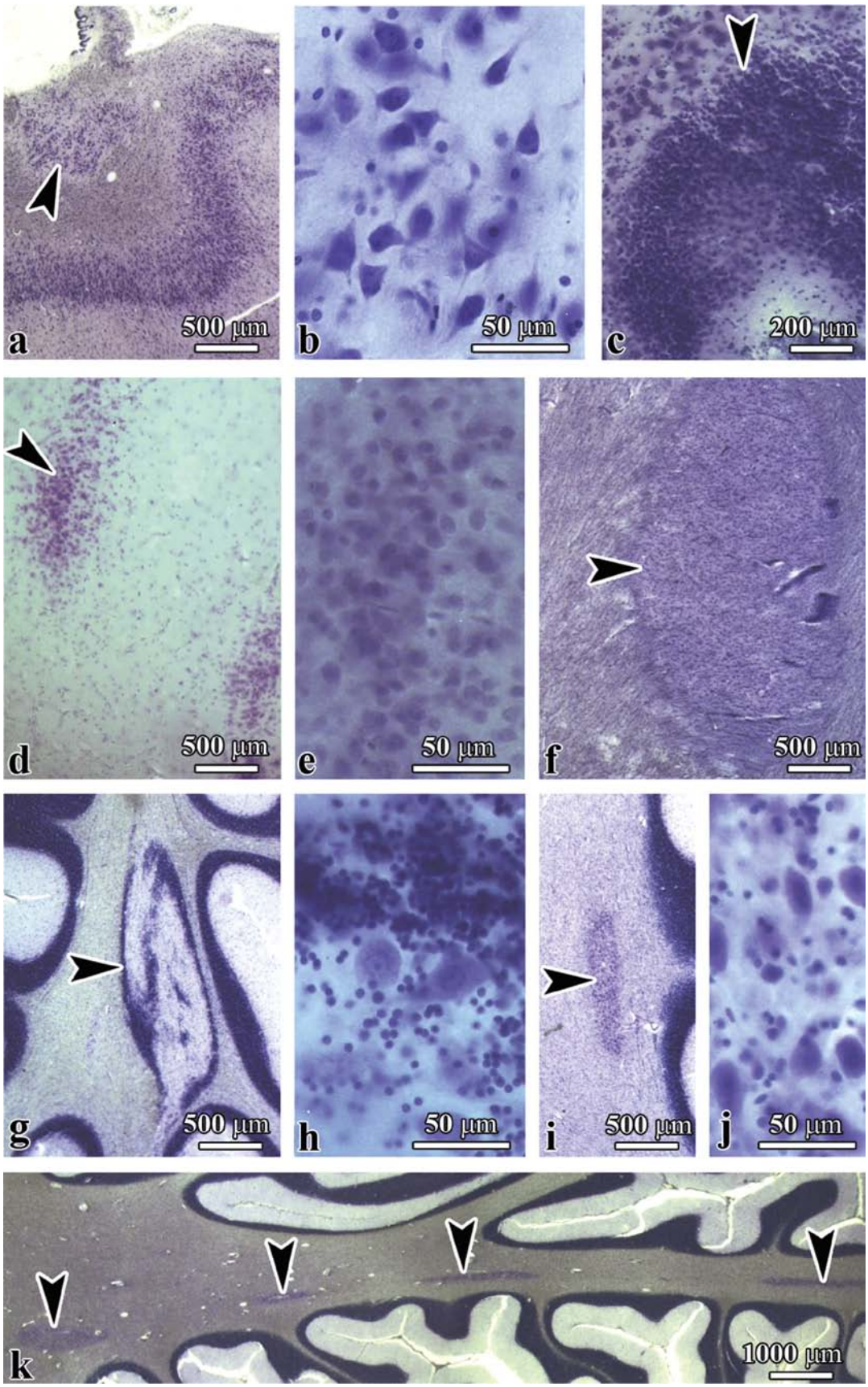

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1 subject had small cerebellar subependymal heterotopias and 2 cases had dysplastic changes in the cerebellar flocculus and nodulus (Table, Supplemental Digital Content 3, http://links.lww.com/NEN/A325). Although all 9 dup(15) subjects and all 10 subjects with autism had developmental abnormalities, there were significant differences between the dup(15) autism and idiopathic autism cohorts in the number of developmental defects and their distribution (Table 5).

Heterotopias

Migration developmental abnormalities were detected as heterotopias in the hippocampal alveus, the CA4 sector, the dentate gyrus (DG) molecular layer, and the cerebral and cerebellar white matter (Fig. 1). A description of each is given below.

Heterotopias in the Hippocampus

A relatively large proportion of heterotopic cells in the alveus had the morphology of pyramidal neurons, although they were much smaller than neurons in the cornu Ammonis and were spatially disoriented. Heterotopias composed of neurons with the morphology of granule neurons of the DG were detected in the CA4 sector and in the molecular layer of the DG. Heterotopias in the alveus, CA4, and DG were found in 8 dup(15) subjects (89%), 1 subject with idiopathic autism (10%), and no control subjects. This difference between dup(15) autism and idiopathic autism cohorts ($p < 0.001$) and dup(15) and control subjects was highly significant ($p < 0.001$; Table 5). The difference between the idiopathic autism and control groups was not significant.

Heterotopias in the Cerebellar White Matter

The morphology of the cerebellar heterotopias reflected 2 types of migration defects. The presence of a mixture of granule and Purkinje cells suggests that clusters of cerebellar cortical neurons do not reach their destination site (Type 1). The second type of cerebellar heterotopia (Type 2) is composed of 1 type of cells with the morphology of cerebellar deep nuclei neurons. Both types of heterotopias were composed of neuronal nuclear marker–positive cells mixed with glial fibrillary acidic protein–positive astrocytes (not shown). In some cases, multiple Type 1 and 2 heterotopias were detected in the cerebellar white matter. In contrast to the significant difference in the prevalence of hippocampal heterotopias, there was no significant difference in the prevalence of heterotopias in the cerebellar white matter between the dup(15) (56%) and idiopathic autism (60%) groups (Table 5). However, the differences between the dup(15) autism and control groups ($p < 0.04$) and between the idiopathic autism and control groups ($p < 0.04$) were significant. Heterotopias in cerebral white matter were rare in both the dup(15) (1/9; 11%) and idiopathic autism (1/10; 10%) groups (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A323, and Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A324).

Dysplasias

Dysplastic changes reflect focal microdysgenesis in the hippocampal DG and cornu Ammonis, the amygdala, and the cerebral and cerebellar cortices. Several types of dysplasia were detected in the DG, including hyperconvolution of the DG, duplication of the granular layer distorting the architecture of the molecular layer of the DG, irregular protrusions of the granular layer into the molecular layer, focal thinning and/or thickening of the granular layer, and fragmentation of the granular layer with the formation of isolated nests of granule cells (Fig. 2). The susceptibility of the DG to developmental abnormalities was several times more apparent in dup(15) syndrome than in idiopathic autism cases. They were detected in 8 (89%) of 9 subjects in the dup(15) group and in only 1 subject with autism (1/10, 10%; $p < 0.001$; Table 5). The number of different types of developmental abnormalities in the dup(15) group ranged from 2 per case in 4 subjects, to 3 per case in 3 subjects, and 5 types in 1 case. The total number of different types of dysplasia was 22 times greater in the dup(15) cohort than in the idiopathic autism cohort ($p < 0.001$). The difference between idiopathic autism (1 positive case) and the control group (no dysplastic changes in the DG) was not significant.

The spectrum of dysplastic changes in the cornu Ammonis comprised abnormal convolution of the CA1 sector, focal deficits of pyramidal neurons, and distortion of the shape, size, and spatial orientation of pyramidal neurons, clustering of dysplastic neurons in the CA1 sector, and many foci of severe microdysgenesis in the CA4 sector, with clustering of immature neurons (Figs. 3A–E). Dysplastic changes in the amygdala resulted in multiple irregular nests of 20 to 40 cells composed of relatively few small immature neurons and numerous oval or bipolar hyperchromatic neurons that were larger than normal amygdala neurons (Fig. 3F). Dysplastic changes in the cornu Ammonis were detected in 2 subjects with dup(15) syndrome and in 2 brains of subjects with autism (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A323, and Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A324).

The presence of dysplastic changes in the cerebral cortex of 5 of the 10 subjects with idiopathic autism (50%) was in striking contrast to the absence of these changes in the dup(15) ($p < 0.03$) and control subjects ($p < 0.04$; Table 5). Three types of cerebral cortex dysplasia were found in the

FIGURE 1. Topography and morphology of heterotopias in the brains of individuals with duplications of chromosome 15q11.2-q13 (subjects with dup[15] and autism). **(A, B)** Heterotopia (**A**, arrowhead) in the alveus composed of bipolar, multipolar, and pyramidal-like neurons shown at higher magnification in **B**. **(C–E)** Heterotopias composed of cells with the morphology of granule neurons in the CA4 sector (**C,** arrowhead) and in the molecular layer (arrowhead) of the dentate gyrus **(D)**; higher magnification is shown in **E**. **(F)** Heterotopia (arrowhead) in the cerebral white matter. **(G–J)** Heterotopias in the cerebellum consist of mixed components of the cerebellar cortex (**G**, arrowhead), including granule and Purkinje cells **(H)**; one is composed of one type of neuron (**I**, arrowhead), with the morphology of cells of cerebellar deep nuclei **(J)**. **(K)** Multifocal heterotopias of both types in cerebellar white matter (arrowheads). **(A–C, F)** dup(15), Case 2; **(D, E)** dup(15), Case 6; **(G–J)** dup(15), Case 5; **(K)** idiopathic autism, Case 5.

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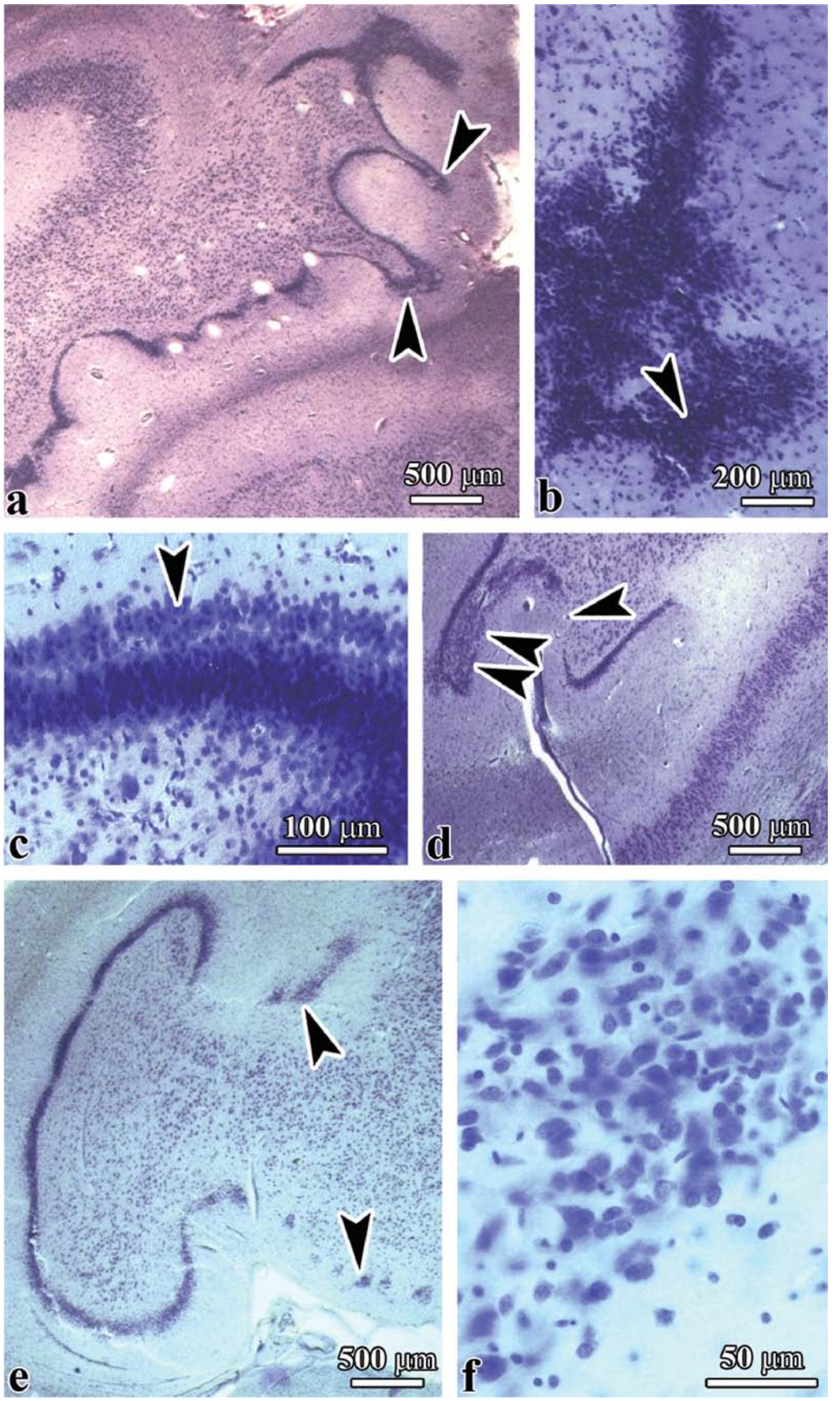

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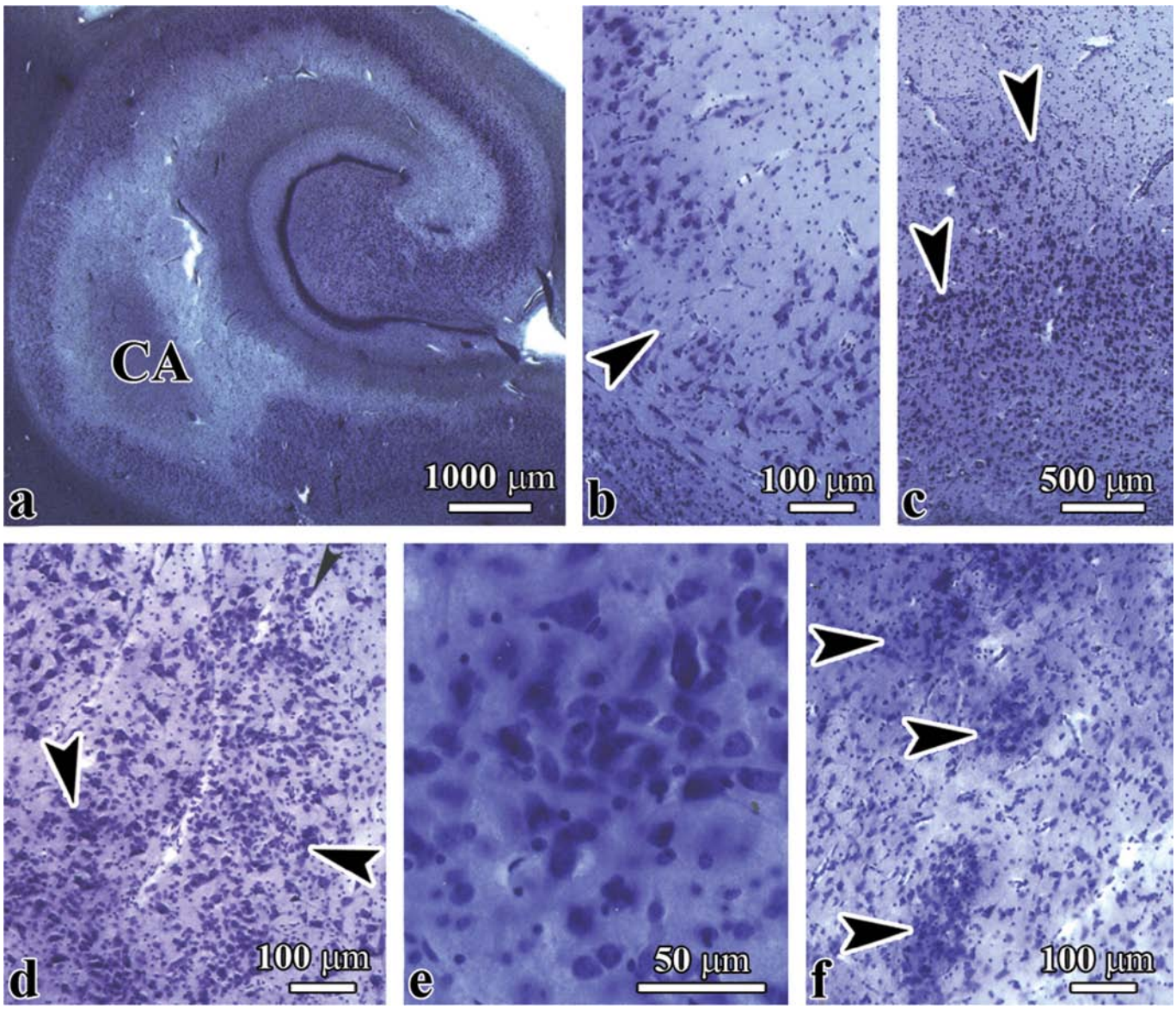

FIGURE 3. Multiple dysplastic changes in the cornu Ammonis (CA) and amygdala in an 11-year-old boy with hexasomy of chromosome 15q11.2q13 (Case 3). **(A–C)** There is abnormal convolution of the CA1 sector **(A)** with focal microdysgenesis of the pyramidal layer **(B**, arrowhead**)** and clustering of disoriented polymorphic neurons **(C**, arrowheads**)**. **(D, E)** Marked multifocal microdysgenesis in the CA4 sector **(D**, arrowheads**)**, with clustering of a mixture of small and large polymorphic neurons **(E)**. **(F)** Multifocal microdysgenesis (arrowheads) in the amygdala is composed of small immature neurons and neurons that are larger than normal amygdala neurons.

idiopathic autism group: focal polymicrogyria, multifocal cortical dysplasia, and bottom-of-a-sulcus dysplasia (Supplemental Digital Content 2, http://links.lww.com/NEN/A324).

Focal polymicrogyria, which reflects a gyrification defect, was found in the frontal lobe in an 8-year-old boy with autism diagnosed with ASD (Case 4), which resulted in local abnormal folding of the cortex with formation of numerous small and irregular microgyri and distortion of the cortical thickness and vertical/horizontal cytoarchitecture (Fig. 4A). The most common developmental abnormality was cortical

FIGURE 2. Six types of dysplastic changes in the dentate gyrus of subjects diagnosed with duplications of chromosome 15q11.2-q13 (dup(15]) syndrome. **(A–F)** Hyperconvolution of the dentate gyrus within the hippocampal body **(A)**, irregular large protrusions of the granular layer **(B)**, duplication of the granular layer **(C)**, focal thinning and discontinuity of granular layer (arrowhead), and thickening of the granular layer confirmed by examination of serial sections (**D**, 2 arrowheads), hippocampal malrotation and granular layer fragmentation into small clusters of cells of irregular shape and variable size **(E, F)**. **(A)** dup(15), Case 8; **(B)** dup(15), Case 7; **(C, E, F)** dup(15), Case 3.

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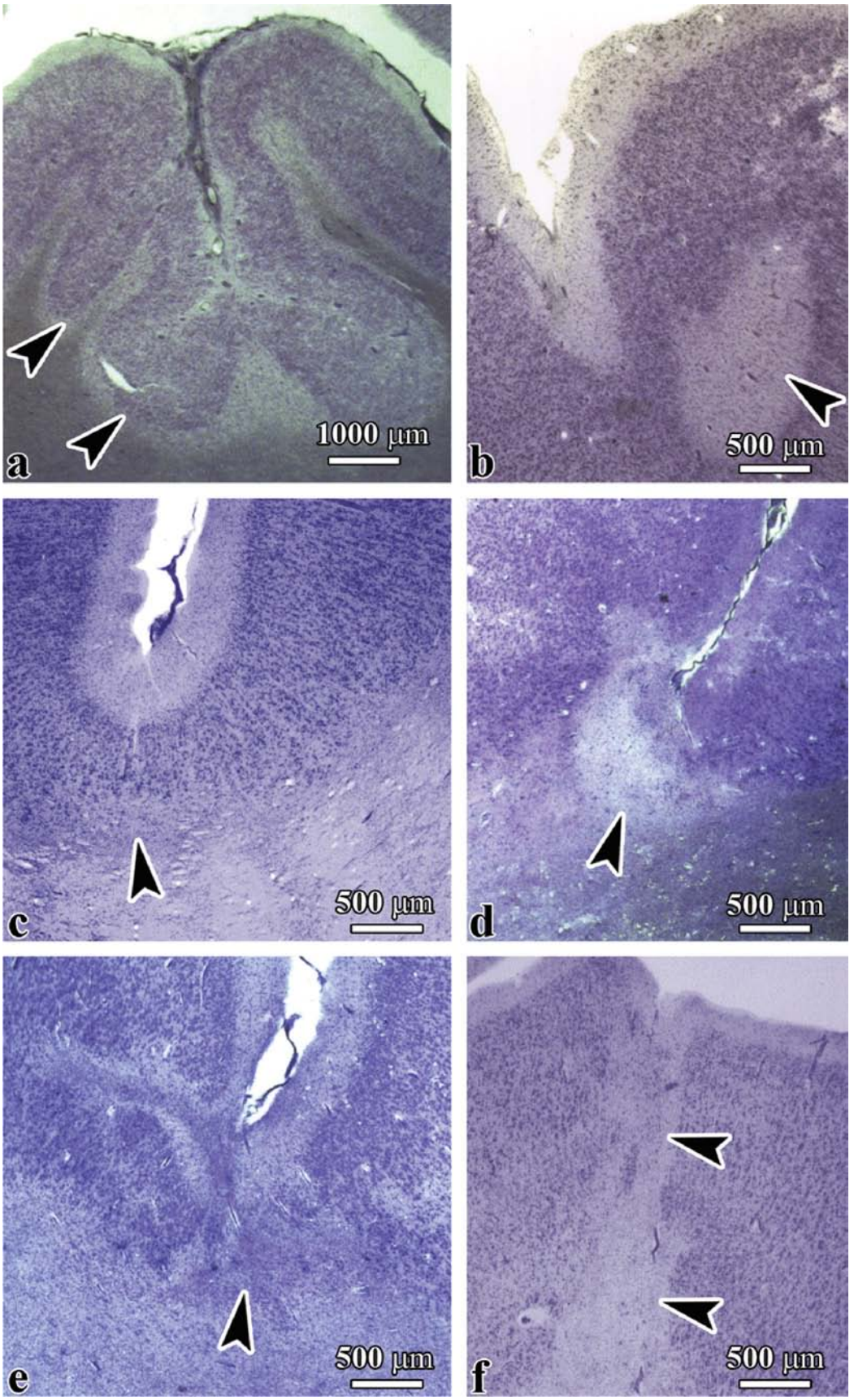

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dysplasia with focal hypocellularity or acellularity and loss of cortical vertical and horizontal cytoarchitecture (Fig. 4B).

Another observed gyrification defect was multifocal bottom-of a-sulcus dysplasia with selective changes in the deepest layer, expansion of dysplastic changes to 2 to 3 deep layers of the cortex, or affecting the entire thickness of the cortex. This developmental abnormality was most often seen in the superior frontal and temporal gyrus, the Heschl gyrus, the middle temporal gyrus, the insula, and the parahippocampal gyrus in a 5-year-old girl diagnosed with idiopathic autism (Case 2; Figs. 4C–F).

Three types of dysplastic changes were found in the cerebellum of the dup(15) subjects and the subjects with autism. These included dysplasia in parts of the nodulus and flocculus, vermis dysplasia, and focal polymicrogyria (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A323, and Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A324). In the nodulus and flocculus, dysplasia resulted in total spatial disorganization of the granular, molecular, and Purkinje cell layer; only a few small abnormally branched Purkinje cells were found to be dispersed among the granule cells in the affected areas. There were many interindividual differences observed in the nodulus or flocculus volume affected by dysplastic changes. Cerebellar dysplasia was commonly observed in both cohorts. Nodulus dysplasia was present in 7 (87%) of 8 dup(15) subjects and in 6 (75%) of 8 subjects with autism (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A323, and Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A324). Flocculus dysplasia was detected in 6 (75%) of 8 dup(15) subjects, in 4 (50%) of 8 subjects with autism, and in 1 (14%) of 7 control subjects (Table 5). The difference between the groups with autism was not significant, but the difference between the dup(15) autism and control group was significant ($p < 0.05$).

Subependymal Nodular Dysplasia

Nodular dysplasia was found in the brain of a 15-year-old adolescent girl diagnosed with dup(15) (Case 5). This consisted of a single large nodule in the wall of the temporal horn of the lateral ventricle and numerous nodules in the wall of the lateral ventricle in the occipital lobe. Subependymal nodular dysplasia was also detected in the brain of a 39-year-old woman diagnosed with dup(15) (Case 9; Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A323). There were numerous subependymal nodules less than 1 to 3 mm in diameter in the wall of the occipital horn of the lateral ventricle in a 32-year-old subject with idiopathic autism (Case 8; Table, Supplemental Digital Content 2, http://links.lww.com/NEN/A324). These were composed of dysplastic neurons with a partially modified morphology of pyramidal, multipolar, or bipolar neurons and oval medium and small cells. In all 3 cases, the nodules were free of oval or polygonal giant cells or ballooned glial cells. Examination of the thalamus, caudate, putamen, nucleus accumbens, and globus pallidus did not reveal developmental qualitative abnormalities in these cohorts.

Differences Between the Global Pattern of Developmental Abnormalities in dup(15) and Autism

Although all dup(15) and subjects with autism had developmental abnormalities, the number of different types of developmental alterations detected in the brains of the dup(15) group was, on average, 2.3 times greater (6.9 per case; $n = 9$) than in the subjects with autism (3 per case; $n = 10$). Analysis of developmental alterations in 13 subjects with autism previously reported (49) revealed developmental abnormalities in the brains of all subjects with autism and a similar prevalence of alterations.

Other Neuropathologic Changes

Selective and marked neuronal loss without gliosis was found in the pyramidal layer in the CA1 sector in the brain of a 10-year-old boy with epilepsy (dup[15]; Case 2). Pathologic alterations extended from the head to the tail of the hippocampal formation, with loss of neurons in the range of 80% in the head and 50% in the body and tail. These findings might be the result of severe and frequent seizures.

An area of marked subpial gliosis was found within a sulcus between the inferior frontal and the orbital gyri in the brain of a dup(15) female with epilepsy and seizure-related asphyxia at the age of 26 years (Case 8). Almost complete focal loss of the granular layer was associated with gliosis, thickening of the affected molecular layer, degeneration of astrocytes, and deposition of many corpora amylacea. These findings most likely represent Chaslin gliosis, indicative of epilepsy-related brain damage. This pathologic condition coexisted with hyperconvolution of the DG, focal thinning, and duplication of the granular cell layer, considered developmental abnormalities contributing to abnormal electrical activity and seizures.

DISCUSSION

Knowledge of the clinical phenotype and genetic factors in autism is based on examination of thousands of individuals with idiopathic autism; however, between 1980 and 2003, only 58 brains of individuals with idiopathic autism were examined postmortem (50). Knowledge of the clinical and genetic characteristics of the dup(15) syndrome is based on examination between 1994 and 2006 of approximately 160 cases (47, 51–54), but the neuropathology of dup(15) with and without autism has not been studied. Results of the application of an extended neuropathologic protocol were previously reported for 13 brains of subjects with idiopathic autism (49). The current study characterizes qualitative neuropathologic changes in the brains of 9 individuals with

FIGURE 4. Cerebral cortex dysplasia autism spectrum disorder (ASD). **(A, B)** Polymicrogyria (**A**, arrowheads), focal hypocellularity or acellularity with lack of vertical and horizontal cortical organization (**B**, arrowhead) in an 8-year-old boy with ASD. **(C–F)** Bottom-of-a-sulcus dysplasia in a 5-year-old girl with idiopathic autism (Case 2). Focal dysplasia was limited to deep cortical layers **(C)**, affected all layers except the molecular layer **(D)**, and caused cortex fragmentation **(E)** or disruption of cortical ribbon continuity (**F**, arrowheads).

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dup(15), including 7 diagnosed as having an ASD (78%). This autism prevalence is in the highest range reported in clinical studies. The association with autism in some of the earlier individual reports (i.e. 4 [33%] of 12 [55] or 6 [36%] of 17) was not based on use of standardized screening (56). A standardized assessment of autistic manifestations in 29 children and adults with a supernumerary idic(15) detected in 20 individuals (69%) with a high probability of ASD (29). All studies reported a significant variability in the autistic phenotype, severity of autistic features, delayed development and/or ID, and seizures among subjects with dup(15) (15).

Major Neuropathologic Differences Between dup(15) Autism and Idiopathic Autism

Numerous studies indicate that autism is associated with a short period of increased brain size (57–59) and more neurons (60). Macrocephaly was detected in 37% of children with autism younger than 4 years (61) and in 42% of the 19 twins diagnosed with idiopathic autism younger than 16 years (6). Postmortem studies (62, 63) and imaging studies (64) also provided converging evidence of increased brain volume in autism. Microcephaly has been observed in only 15.1% of 126 children with autism aged 2 to 16 years (65) and is usually associated with severe pathology (66, 67), ID, and other medical disorders (65).

This study revealed a high prevalence of microcephaly in the dup(15) autism cohort examined postmortem, that is, the mean weight of the brains of subjects with dup(15) autism was 300 g less than that of subjects with idiopathic autism and 189 g less than that in the controls ($p < 0.001$ for both). The characteristics of head circumference and brain volume in dup(15) cohorts have been studied less comprehensively than in idiopathic autism, but published reports also show a strong prevalence of microcephaly. A summary of records from 107 supernumerary inv dup(15) cases revealed that only 3 subjects had macrocephaly (2.8%), but 6 times more cases ($n = 18$, 16.8%) had microcephaly (15). Battaglia (68) detected microcephaly in radiologic evaluations of 1 in 4 subjects with dup(15) whose age ranged from 4 to 8 years. These data suggest the failure of mechanisms controlling brain growth in autism, resulting in the prevalence of macrocephaly in idiopathic autism and of microcephaly in dup(15) autism.

Our extended neuropathologic protocol revealed several striking differences between the pattern of developmental alterations in dup(15) autism and idiopathic autism. Neuronal migration defects in the hippocampus resulting in heterotopias in the alveus, CA4, and DG were 8 times more common in dup(15) autism (89% of subjects) than in idiopathic autism (10%; $p < 0.001$). The second developmental abnormality distinguishing dup(15) autism from idiopathic autism was the dysplasia that occurred 8 times more often in the DG of subjects with dup(15) and the different types of developmental abnormalities that occurred 22 times more often in the DG of subjects with dup(15) ($p < 0.001$). The third factor differentiating these 2 cohorts was the absence of cerebral cortex dysplasia in dup(15) autism and the presence of this pathology in 50% of subjects with idiopathic autism ($p < 0.03$). The increased number of developmental alterations and the topographic differences suggest significant differences between mechanisms contributing to abnormal neuronal migration and altered cytoarchitecture in these 2 cohorts.

Linkage and gene mapping analysis, molecular reports, and clinical studies revealed the link between de novo, maternally derived proximal 15q chromosome alterations, and autism (13, 15, 27, 33, 56, 69–72). This postmortem study suggests that neuropathologic profile with microcephaly and multiple focal developmental defects is another marker of maternally derived proximal 15q chromosome alterations contributing to autistic phenotype.

Although ASD and epilepsy are heterogeneous disorders, they often occur together. This may indicate that these disorders share some underlying mechanisms and that epileptogenesis affects brain structure and function, which modify the clinical manifestations of autism. Approximately 30% of children with autism are diagnosed with epilepsy and 30% of children with epilepsy are diagnosed with autism (73). Significant cognitive impairment is present in approximately 50% of all individuals who have autism (74). Early onset of seizures contributes to clinical regression, enhanced severity of autistic phenotype, and enhanced mortality. The rate of death among subjects with autism is 5.6 times higher than expected (75), and epilepsy- and cognitive impairment–related accidents account for most of the deaths (75–78). The diagnosis of epilepsy in 78% (7/9) of postmortem-examined individuals with dup(15) indicates that epilepsy is an important component of the clinical phenotype in most individuals diagnosed with dup(15).

Microcephaly might be one of several indications of brain immaturity that increases the risk of epilepsy. The immature brain exhibits increased excitation, diminished inhibition, and increased propensity for seizures in infancy and early childhood (79). The reduced volume of neurons in most subcortical structures and some cortical regions in the brains of 4- to 8-year-old children with autism seems to reflect brain immaturity in early childhood (80). In the normally developing brain, maturation of the frontal and temporal cortex is associated with differential expression of 174 genes; however, none of these genes are differentially expressed in ASD (81). Altered development of neurons resulting in brain immaturity may contribute to an increased tendency for the seizures and epileptogenesis observed in the examined cohort. Very early onset of intractable epilepsy (at 10, 8, and 10 months, respectively) was reported in all 3 of the youngest individuals diagnosed with dup(15), who died as a result of SUDEP at 9, 10, and 11 years. These findings suggest that very early onset of seizures and very severe seizures may increase the risk of SUDEP in this cohort. Severe ID in all 3 of these individuals indicates that brain immaturity and a profound ID are another SUDEP risk factor in the examined cohort of subjects with dup(15). The combination of all these factors may contribute to death at a very early age (~10 years).

Sudden unexpected death in childhood (SUDC) is the sudden death of a child older than 1 year that, despite a review of the clinical history, circumstances of death, and complete autopsy with appropriate ancillary testing, remains unexplained.

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It occurs throughout childhood (<18 years) but occurs most commonly between the ages of 1 and 4 years (82, 83). Sudden unexpected death in childhood is an unexpected and unexplained death that occurs in patients with known epilepsy, including children, and is typically associated with sleep (84, 85). Kinney et al (86) reported several types of hippocampal anomalies in SUDC cases, including hyperconvolution of the DG, focal duplication of the DG, granular nodular heterotopia, abnormal folding of the subiculum, and focal clustering of pyramidal neurons in the cornu Ammonis. These developmental anomalies are considered a cause of seizure-related autonomic and/or respiratory dysfunction and sudden death (87–89). Kinney et al (86) proposed that these anomalies represent an epileptogenetic focus that, when triggered by fever, trivial infection or minor head trauma at a susceptible age, may result in unwitnessed seizure, cardiopulmonary arrest, and sudden death. The presence of these developmental anomalies in the examined brains of individuals with dup(15) may explain SUDEP in 5 cases and SUDC in 2 other cases. The presence of these changes in the hippocampus of subjects with dup(15) who died of causes other than SUDEP or SUDC suggests that they also were at higher risk for sudden unexpected death. Collectively, these data indicate that the risk of SUDC is much higher in the dup(15) cohort than in the general population with SUDC in which there is an overall rate of 57 of 100,000 deaths per year (90).

Microdysgenesis is not specific for dup(15), epilepsy, or autism; it has been reported in ID (91), psychosis (92), and dyslexia (93), as well as in some control subjects. None of these developmental alterations can be considered pathognomonic of an ''epileptic'' brain (83), but changes in the cohort affected by dup(15) or idiopathic autism reveal significant differences. This indicates that not a single lesion but, instead, a complex pattern of developmental defects distinguishes these subjects. A 2.3-fold higher prevalence of these developmental abnormalities, 2.3 times higher prevalence of epilepsy, and 6 times higher prevalence of epilepsy-related death in the dup(15) cohort compared with the idiopathic autism group suggest that the mechanisms leading to developmental structural alteration in the hippocampal formation are a major contributor to epilepsy and SUDEP/SUDC in dup(15). These seem to be a much weaker contributor to epilepsy and SUDEP in idiopathic autism. The results presented in this report reinforce the hypothesis that additional copies of the critical 15q11-q13 region are causally related to the autism phenotype and developmental abnormalities contributing to epilepsy and to an increased risk of SUDEP and SUDC. Future studies of the expression and distribution of proteins encoded by $GABA_A$ receptor subunit genes ($\alpha 5$, $\beta 3$, and $\gamma 3$) and the gene for juvenile epilepsy located near D14S165 on chromosome 15 may explain the role of duplication or triplication of these genes in autism and the enhanced susceptibility to seizures in dup(15) syndrome.

In conclusion, despite the common clinical diagnosis of autism in the dup(15) and idiopathic cohorts, significant differences in brain growth and focal developmental defects of neuronal migration and cytoarchitecture indicate that the dup(15) autistic phenotype is a product of unique genetic, molecular, and neuropathologic alterations.

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Supplemental Table 1. **Developmental Alterations in the Brain of Subjects Diagnosed with dup (15)**

Case #	Sex, Age	**Developmental Alterations. [Other neuropathological changes listed in square brackets].**
1	M, 9	Heterotopias in the alveus and stratum oriens in the hippocampal head and body (1). Heterotopia in the cerebellar white matter (2). Dysplasia of the dentate gyrus with local granule cell layer thinning (3) and fragmentation into numerous separate neuronal islands (4). Dysplasia in the cerebellar nodulus (5).
2	M, 10	Heterotopias in the alveus (1). Dentate gyrus granule cell layer discontinuity (2) and hyperconvolution (3). Heterotopias in the cerebral white matter (4). Dysplasia in the cerebellar nodulus (5) and flocculus (6). [Severe neuronal loss in the CA1].
3	M, 11	Multiple foci of microdysgenesis in amygdala (1). Heterotopia in the hippocampal alveus (2). Fragmentation of the granule cell layer of the dentate gyrus (3) and focal duplication of the granule cell layer (4). Abnormal convolution and malrotation of the cornu Ammonis (5). Focal microdysgenesis of the pyramidal layer with a deficit of pyramidal neurons (6) and clustering of disoriented immature neurons in CA1 (7). Severe multifocal microdysgenesis in the CA4 sector with clustering of small and large polymorphic neurons (8). Two heterotopias built of granule and Purkinje cells in the cerebellar white matter (9). Dysplasia in the cerebellar nodulus (10) and flocculus (11).
4	F, 15	Heterotopia in the alveus (1). Dysplasia in cerebellar nodulus (2) and flocculus (3).
5	F, 15	Heterotopia in the alveus (1). Dentate gyrus microdysgenesis with focal duplication or triplication of granule cell layer (2), discontinuity of granule layer (3), and granule cell layer several-fold thickening (4). Large subependymal nodular dysplasia in lateral ventricle (5). Multifocal subependymal nodular dysplasia within the wall of the ventricle in the occipital lobe (6). Cerebellar developmental alterations with focal polymicrogyria (7), small heterotopia built of cells resembling neurons of the cerebellar deep nuclei (8), and large heterotopia build of granule neurons and Purkinje cells (9).
6	M, 20	Heterotopia in the dentate gyrus molecular layer (1), focal granule layer thinning and fragmentation (2), and duplication (3). Dysplasia in the cerebellar nodulus (4) and flocculus (5). [Severe hypoxic encephalopathy].
7	M, 24	Dentate gyrus hyperconvolution (1). Dentate gyrus granule cell layer duplication (2), irregular large protrusions (3), focal thinning and discontinuity (4), and focal fragmentation (5). Two large heterotopias in the CA4 sector built of cells with morphology of granule layer neurons (6). Multi-focal microdysgenesis in the CA4 sector with large and small clusters of immature small neurons (7). Two types of heterotopias in the cerebellar white matter built of neurons resembling cerebellar nuclei (8) or altered cerebellar cortex (9). Dysplasia in the cerebellar nodulus (10) and flocculus (11).
8	F, 26	Hyperconvolution of the dentate gyrus (1). Focal thinning (2) and duplication (3) of the dentate gyrus granule cell layer. Cerebellar developmental changes with multiple focal microdysgenesis in the vermis (4), dysplasia in the cerebellar nodulus (5) and flocculus (6), and subcortical and periventricular heterotopias in the cerebellar white matter (7). [Seizure-related focal loss of neurons in the second layer of the orbital and frontal inferior gyrus with Chaslin gliosis].
9	F, 39	Hyperconvolution of the dentate gyrus (1). Focal thinning (2) and thickening (3) of the granule cell layer in the dentate gyrus. Heterotopia in the CA4 sector (4). Heterotopias in the CA1 sector (5). Subependymal nodular dysplasia (6). [Multifocal calcification in basal ganglia and white matter].

Supplemental Table 2. Developmental Alterations in the Brains of Subjects Diagnosed with Idiopathic Autism

Case #	Sex, **Age**	**Developmental Alterations. [Other neuropathological changes listed in brackets]**
1	M, 2	Frontal lobe dysplasia type I with abnormal thickening of the cortex, focal loss of the molecular and thickening of the granule layer, and loss of the vertical and horizontal organization (1). Duplication of the dentate gyrus granule cell layer (2). Multifocal cerebellar heterotopias (3). Dysplasia in the cerebellar nodulus (4) and flocculus (5). [Severe multifocal necrosis caused by hypoxic-ischemic changes associated with mechanism of death].
2	F, 5	Distorted cortical gyrification with severe multifocal neocortical dysplasia in the superior frontal and temporal gyrus, insula, Heschl gyrus, middle temporal g, and parahippocampal gyrus. Microdysgenesis of deep cortical layers especially often and severe at the bottom of cortical sulcus (2). Cerebellar floccular dysplasia (3).
3	M, 5	Multifocal cortical dysplasia type I in the parietal and occipital lobes with focal hypo- or acellularity and loss of cortical cytoarchitecture (1). Cerebellar floccular (2) and nodular (3) dysplasia.
4	M, 8	Frontal lobe focal polymicrogyria (1). Dysplasia with abnormal lamination in frontal and temporal cortex (2). Heterotopia in alveus (3). Heterotopia in the subependymal area in cerebellar white matter (4). Cerebellar floccular (5) and nodular (6) dysplasia.
5	M, 9	Multiple heterotopias within the cerebellar white matter (1). Cerebellar nodular dysplasia (2).
6	M, 11	Focal ectopic large neurons within folial subcortical white matter (1). [Cardiac arrest encephalopathy with generalized ischemic brain damage].
7	M, 29	Developmental focal neuronal deficit in the temporal cortex (hypocellularity) (1). Two heterotopias in the cerebellar white matter (2). Cerebellar nodular dysplasia (3). [Mild vasculitis].
8	M, 32	Very prominent subependymal nodular dysplasia in the wall of the occipital horn of lateral ventricle (no giant or bizarre cells) (1). Heterotopia in the periventricular white matter near frontal horn of the lateral ventricle (2). Hyperconvolutions of the CA1 and dentate gyrus (3). Heterotopia in the cerebellum (4). [Glioblastoma multiforme of the right temporo-occipital-parietal area].
9	M, 51	Nodular dysplasia (1). [Cardiac arrest encephalopathy with generalized ischemic brain damage].
10	M, 52	Hyperconvolution and distortion of laminar organization of the CA1 (1). Distortion of laminar organization of the subiculum proper (2).

Supplemental Table 3. Neuropathology in the Control Group

Case #	Sex	Age, Years	Developmental Alterations [Other Neuropathological Changes Listed in Brackets]
1	F	8	[Focal loss or deficit of neurons without glial response in the caudate nucleus and temporal cortex]
2	M	14	No pathological changes
3	M	14	No pathological changes
4	M	32	Subependymal heterotopia in the cerebellum (1). [Perivascular calcifications in the hippocampus and in the globus pallidus]
5	F	33	No pathological changes
6	F	43	Cerebellar nodular dysplasia (1).
7	M	47	Cerebellar nodular (1) and floccular (2) dysplasia.

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Abnormal Intracellular Accumulation and Extracellular Aβ Deposition in Idiopathic and Dup15q11.2-q13 Autism Spectrum Disorders

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Abstract

Background: It has been shown that amyloid ß (Aβ), a product of proteolytic cleavage of the amyloid β precursor protein (APP), accumulates in neuronal cytoplasm in non-affected individuals in a cell type–specific amount.

Methodology/Principal Findings: In the present study, we found that the percentage of amyloid-positive neurons increases in subjects diagnosed with idiopathic autism and subjects diagnosed with duplication 15q11.2-q13 (dup15) and autism spectrum disorder (ASD). In spite of interindividual differences within each examined group, levels of intraneuronal Aβ load were significantly greater in the dup(15) autism group than in either the control or the idiopathic autism group in 11 of 12 examined regions ($p<0.0001$ for all comparisons; Kruskall-Wallis test). In eight regions, intraneuronal Aβ load differed significantly between idiopathic autism and control groups ($p<0.0001$). The intraneuronal Aβ was mainly N-terminally truncated. Increased intraneuronal accumulation of $Aβ_{17–40/42}$ in children and adults suggests a life-long enhancement of APP processing with α-secretase in autistic subjects. Aβ accumulation in neuronal endosomes, autophagic vacuoles, Lamp1-positive lysosomes and lipofuscin, as revealed by confocal microscopy, indicates that products of enhanced α-secretase processing accumulate in organelles involved in proteolysis and storage of metabolic remnants. Diffuse plaques containing $Aβ_{1–40/42}$ detected in three subjects with ASD, 39 to 52 years of age, suggest that there is an age-associated risk of alterations of APP processing with an intraneuronal accumulation of a short form of Aβ and an extracellular deposition of full-length Aβ in nonfibrillar plaques.

Conclusions/Significance: The higher prevalence of excessive Aβ accumulation in neurons in individuals with early onset of intractable seizures, and with a high risk of sudden unexpected death in epilepsy in autistic subjects with dup(15) compared to subjects with idiopathic ASD, supports the concept of mechanistic and functional links between autism, epilepsy and alterations of APP processing leading to neuronal and astrocytic Aβ accumulation and diffuse plaque formation.

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Introduction

Autism is a developmental disorder characterized by qualitative impairments in reciprocal social interactions, verbal and nonverbal communication, and restricted, repetitive and stereotyped patterns of behavior [1]. Autism is often diagnosed in subjects with genetic disorders, including maternal origin duplications 15q11.2-q13

(dup15) (69%) [2,3], fragile X syndrome (FXS) (15–28%) [4] and Down syndrome (DS) (7%) [5].

Recent studies indicate that non-amyloidogenic cleavage of the amyloid-β (Aβ) peptide precursor (APP) with α and γ secretases is linked to several developmental disorders, including autism and FXS [6–10]. The proteolytic cleavage of APP by membrane-associated secretases releases several Aβ peptides possessing heterogeneous amino- and carboxyl-terminal residues, including $A\beta_{1-40}$ and $A\beta_{1-42}$ as products of β- and γ-secretases (amyloidogenic pathway); $A\beta_{17-40/42}$, as a product of α- and γ-secretases (p3 peptide, non-amyloidogenic pathway) [11,12]; and $A\beta_{pE3}$ as a product of N-terminal truncation of full-length Aβ peptide by aminopeptidase A and pyroglutamate modification [13]. Aβ peptides differ in toxicity, oligomerization, fibrillization, distribution and trafficking within cells, and in their contribution to Aβ deposits in plaques and vascular walls. Alzheimer disease (AD) is associated with oligomeric Aβ accumulation, fibrillar Aβ deposition in plaques, neuronal degeneration and cognitive decline. Intraneuronal Aβ accumulation has been shown to be an early event in AD brains and in transgenic mouse models of AD, and has been linked to synaptic pathology [14,15].

Detection of significantly increased levels of sAPP-α in blood plasma in 60% of autistic children was reported to be an early biomarker of a subgroup of children with autism [6]. Enhanced APP processing by α-secretase is especially prominent in autistic subjects with aggressive behavior [6,16]. Sokol et al. [10] proposed that increased levels of sAPP-α contribute to both the autistic and FXS phenotypes, and that excessively expressed sAPP-α neurotrophic activity may contribute to an abnormal acceleration of brain growth in autistic children and to macrocephaly in FXS. The fragile X mental retardation protein (FMRP) binds to and represses the dendritic translation of APP mRNA, and the absence of FMRP in FXS and in *Fmr1*^{KO} mice results in the upregulation of APP, $A\beta_{40}$ and $A\beta_{42}$ [7]. Westmark et al. [8] also revealed that genetic reduction of *AβPP* by removal of one *App* allele in *Fmr1*^{KO} mice results in reversion of FXS phenotypes, including reduction of plasma $A\beta_{1-42}$, to normal levels. Experimental studies in *Fmr1*^{KO} mice [17] suggest that over-expression of APP/Aβ may contribute to the seizures observed in autism [18] and FXS [4] and that both the over- and under-expression of APP and its metabolites increase the incidence of seizures [7,17,19,20].

Previously we reported that in the brains of controls, both children and adults, neurons accumulate cell type–specific amounts of $A\beta_{17-40/42}$, which is the product of nonamyloidogenic APP processing [21]. One may hypothesize that increased levels of sAPP-α in blood plasma [6,9,16] reflect the enhanced non-amyloidogenic processing of neuronal APP with α-secretase in the brain of autistic subjects.

The aims of this comparative study of the brains of subjects with idiopathic autism (autism of unknown etiology) and autism caused by maternal origin dup(15) were (a) to test the hypothesis that regardless of the causative mechanism, autism is associated with an enhanced accumulation of Aβ in neuronal cytoplasm, (b) to show that intraneuronal Aβ is the product of non-amyloidogenic α-secretase APP cleavage ($A\beta_{17-40/42}$), (c) to show brain region– and cell type–specific Aβ immunoreactivity, and (d) to identify cytoplasmic organelles involved in Aβ accumulation in the neurons of autistic and control subjects.

Results

The Difference between Intraneuronal Aβ Accumulation in dup(15) Autism, Idiopathic Autism and Control Groups

In all subjects with dup15/autism spectrum disorder (ASD) and the majority of individuals with idiopathic ASD, intraneuronal Aβ immunoreactivity was observed in more neurons, and the amount of immunoreactive material was increased in comparison to the control subjects (Fig. 1). The morphology of the intracellular deposits of Aβ-positive material was cell type–specific. Cortical pyramidal neurons showed significant heterogeneity of intraneuronal deposits with a mixture of fine granular material and several times larger 4G8-positive granules. In Purkinje cells, fine granular deposits were accumulated in the cell body. In the dentate nucleus, large neurons accumulated fine granular material, whereas small neurons accumulated a few much larger Aβ-positive granules. Neurons in the reticulate nucleus in the thalamus contained a mixture of fine granular material and large 4G8-positive granules.

Immunocytochemistry with monoclonal antibodies (mAbs) 4G8 (17–24 aa of Aβ) and 6E10 (4–13 aa of Aβ) revealed that almost all intraneuronal Aβ is 4G8-positive, but only a very small proportion is labeled with 6E10.

Quantitative evaluation of 12 brain subregions/cell types (frontal, temporal and occipital cortex, Purkinje cells, amygdala, thalamus, lateral geniculate body (LGB), dentate gyrus, CA1 and CA4 sectors and dentate nucleus) revealed that in 11 subregions intraneuronal Aβ load was significantly greater in the dup(15) autism group than in the control and idiopathic autism cohorts ($p<0.0001$ for all comparisons). In eight regions (all three cortical subregions, Purkinje cells, amygdala, thalamus, LGB, and dentate gyrus), intraneuronal Aβ load differed significantly between the idiopathic autism and control groups ($p<0.0001$). In structures with almost all neurons positive for Aβ–the dentate nucleus and the inferior olive–the amyloid load was insignificantly higher in control subjects than in subjects with idiopathic autism.

Quantitative study revealed different patterns of immunoreactivity in brain subregions (Fig. 2, and Supporting Information, Fig. S1). The characteristic feature distinguishing the amygdala, thalamus and Purkinje cells of subjects with dup(15) autism was the very high percentage of neurons with strong cytoplasmic Aβ immunoreactivity (46%, 46% and 35%, respectively); the percentage was significantly lower in the idiopathic autism group (32%, 38% and 19%, respectively), and very low in control subjects (6%, 6% and 12%, respectively). However, in pyramidal neurons in the frontal, temporal and occipital cortex, the percentage of neurons with strong Aβ immunoreactivity was low (3–10%), whereas the total percentage of Aβ-positive neurons was significantly higher in the dup(15) group (81–83%) than in the idiopathic autism group (56–71%) and in control subjects (45–51%).

The percentage of Aβ-positive neurons and neuronal amyloid load was smaller in the hippocampal formation, especially in the CA1 sector and dentate gyrus of control subjects. The amyloid load was significantly higher in the dup(15) autism group than in control subjects, but the difference in amyloid load between the idiopathic autism and control groups was insignificant (Fig. S1).

The feature distinguishing the LGB, inferior olive and dentate nucleus from other brain structures is the childhood onset of lipofuscin accumulation. In LGB, strong Aβ immunoreactivity was observed in 73% of neurons in dup(15) autism and in 62% in idiopathic autism but only 16% of LGB neurons were strongly Aβ-positive in control subjects. In the dentate nucleus, the percentage of strongly positive neurons was comparable in all three groups (41%, 35% and 41%, respectively), but overall amyloid load was statistically higher in dup(15) autism. The percentage of strongly Aβ-positive neurons in the inferior olive was the same in the idiopathic autism and in the dup(15) (32%) group, and there was no difference in overall amyloid load between autistic and control subjects (Fig. S1).

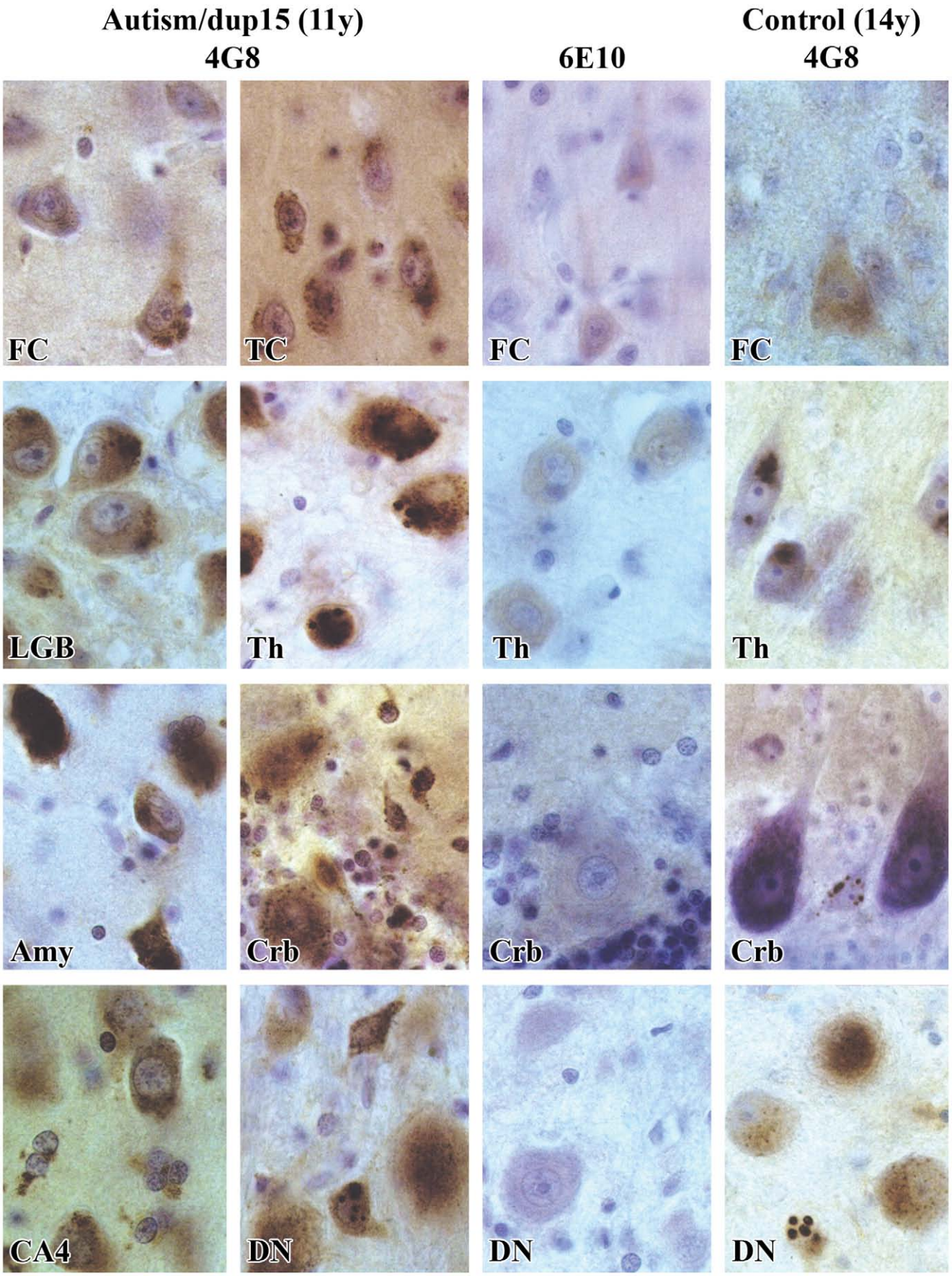
Autism/dup15 (11y)
4G8
6E10
Control (14y)
4G8
FC
TC
FC
FC
LGB
Th
Th
Th
Amy
Crb
Crb
Crb
CA4
DN
DN
DN

Figure 1. Enhanced intraneuronal accumulation of amino-terminally truncated Aβ in autism. Mapping of $A\beta_{17-24}$ in the brain AN09402 reveals brain region– and cell type–specific patterns of abnormal Aβ accumulation in the cytoplasm of neurons and glial cells of a male diagnosed with dup(15), autism and intractable epilepsy, whose sudden unexpected death at the age of 11 years was seizure-related. Almost all neurons in the frontal (FC) and temporal cortex (TC) are 4G8-positive, but the reaction intensity varies from weak to strong. Strong immunoreactivity is observed in many neurons in the lateral geniculate body (LGB), thalamus (Th), amygdala (Amy), Purkinje neurons and basket and stellate neurons in the molecular layer in the cerebellar (Crb) cortex, in many neurons and astrocytes in the CA4, and large and small neurons in the dentate nucleus (DN). Some types of neurons (in the reticular nucleus in the thalamus and small neurons in the dentate nucleus) have different types of deposits: fine-granular and 2- to 3-μm in diameter 4G8-positive deposits. No reaction or only traces of a reaction detected with mAb 6E10 in the frontal cortex, thalamus, cerebellum and dentate nucleus indicate that in intraneuronal Aβ the amino-terminal portion is missing, and the prevalent form of Aβ is α-secretase product. Immunoreactivity with mAb 4G8 is present in the brain of the control subject (14 years of age), but fewer neurons are positive, and immunoreactivity in the frontal cortex, thalamus, cerebellum and dentate nucleus is weaker than in the affected subject. In the control subject, glial cells are usually 4G8-immunonegative.
doi:10.1371/journal.pone.0035414.g001

Aβ in Glial Cells

Astrocytes and microglia in the control brains were usually Aβ-negative or contained only traces of Aβ immunoreactivity. Enhanced neuronal Aβ accumulation in the brains of individuals with autism was associated with Aβ accumulation in the astrocytes' cytoplasm and in some microglial cells (Fig. 3). Two patterns of Aβ immunoreactivity were observed in astroglia. The most common form was a condensed aggregate of Aβ in one pole of the astrocyte soma typical for CA4 sector, some cortical areas but without clear anatomical predilection, and the cerebellar cortex border zone between granule and molecular layers. The less common form was deposition of Aβ-immunoreactive granular material in the entire astrocyte body and in a proximal portion of processes radiating from the cell body (frequent in the molecular layer of the cerebral cortex). The increase in the amount of cytoplasmic Aβ was often paralleled by (a) a several-fold increase in the number of astrocytes, all of which were Aβ-positive (Fig. 3a), (b) clustering of astrocytes in groups of 3–10 cells (Fig. 3b), (c) numerous mitoses as a sign of astrocyte proliferation (Fig. 3c,d) and (d) astrocyte death resulting in deposition of extracellular remnants of Aβ aggregates (Fig. 3e) similar to those seen in astrocyte cytoplasm. Extracellular Aβ deposits were found in neuropil, but larger aggregates (more than 10) were more often in the perivascular space. Confocal microscopy confirmed Aβ accumulation in GFAP-positive astrocytes (Fig. 3, lower panel).

Intracellular Distribution of Amino-terminally Truncated Aβ in Neurons

Intraneuronal Aβ deposits revealed striking neuron type–specific differences in amount, morphology and cytoplasmic

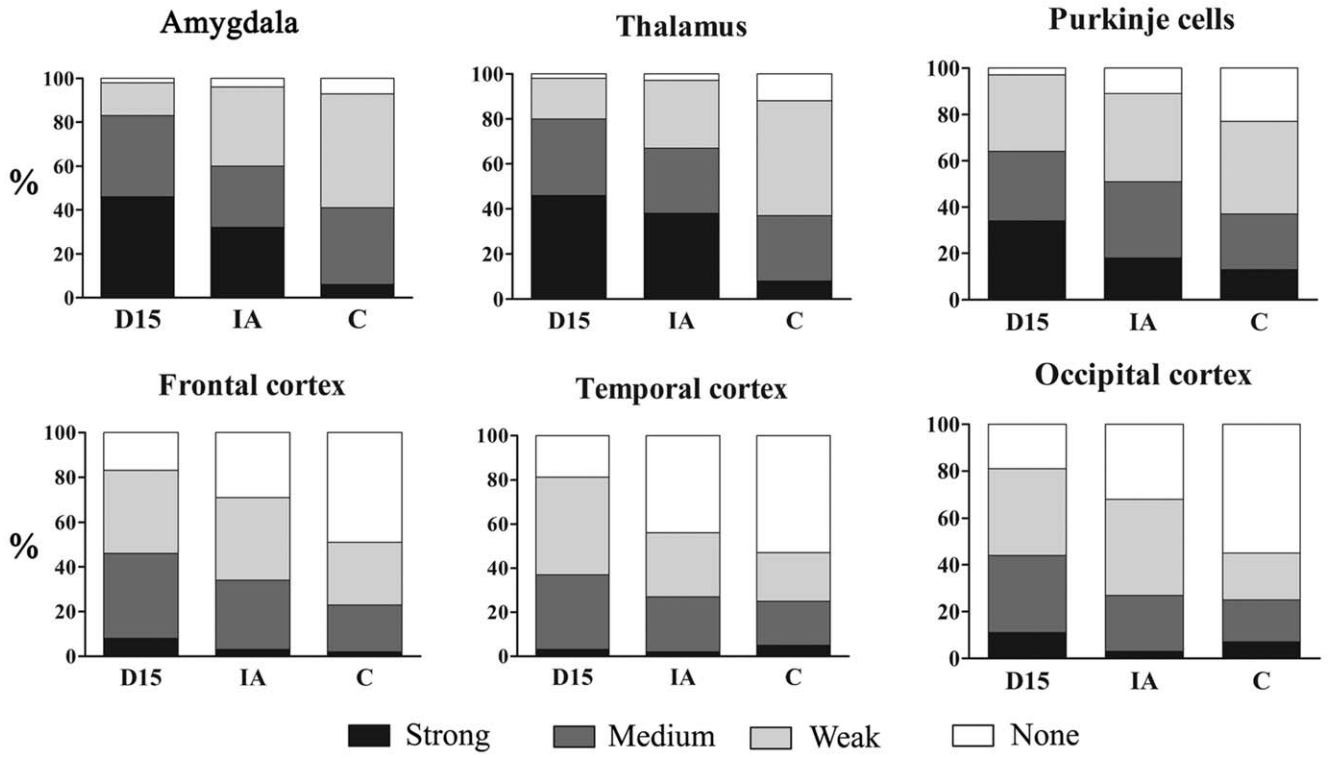

Figure 2. Two major patterns of alterations in intraneuronal Aβ accumulation. Graphs show a high percentage of neurons with strong cytoplasmic immunoreactivity (mAb 4G8) in the amygdala, thalamus and Purkinje cells in subjects diagnosed with dup(15) autism (D15), a lower percentage in idiopathic autism (IA) subjects, and a low percentage in control subjects. In contrast, the characteristic feature of pyramidal neurons in the frontal, temporal and occipital cortex is a low percentage of neurons with strong Aβ immunoreactivity, whereas the total percentage of Aβ-positive neurons is significantly higher in the dup(15) group than in the idiopathic autism group or in control subjects. Differences in Aβ immunoreactivity in the dup(15) autism vs. control cohort, the idiopathic autism vs. control group, and the dup(15) autism vs. idiopathic autism are significant ($p<0.0001$).
doi:10.1371/journal.pone.0035414.g002

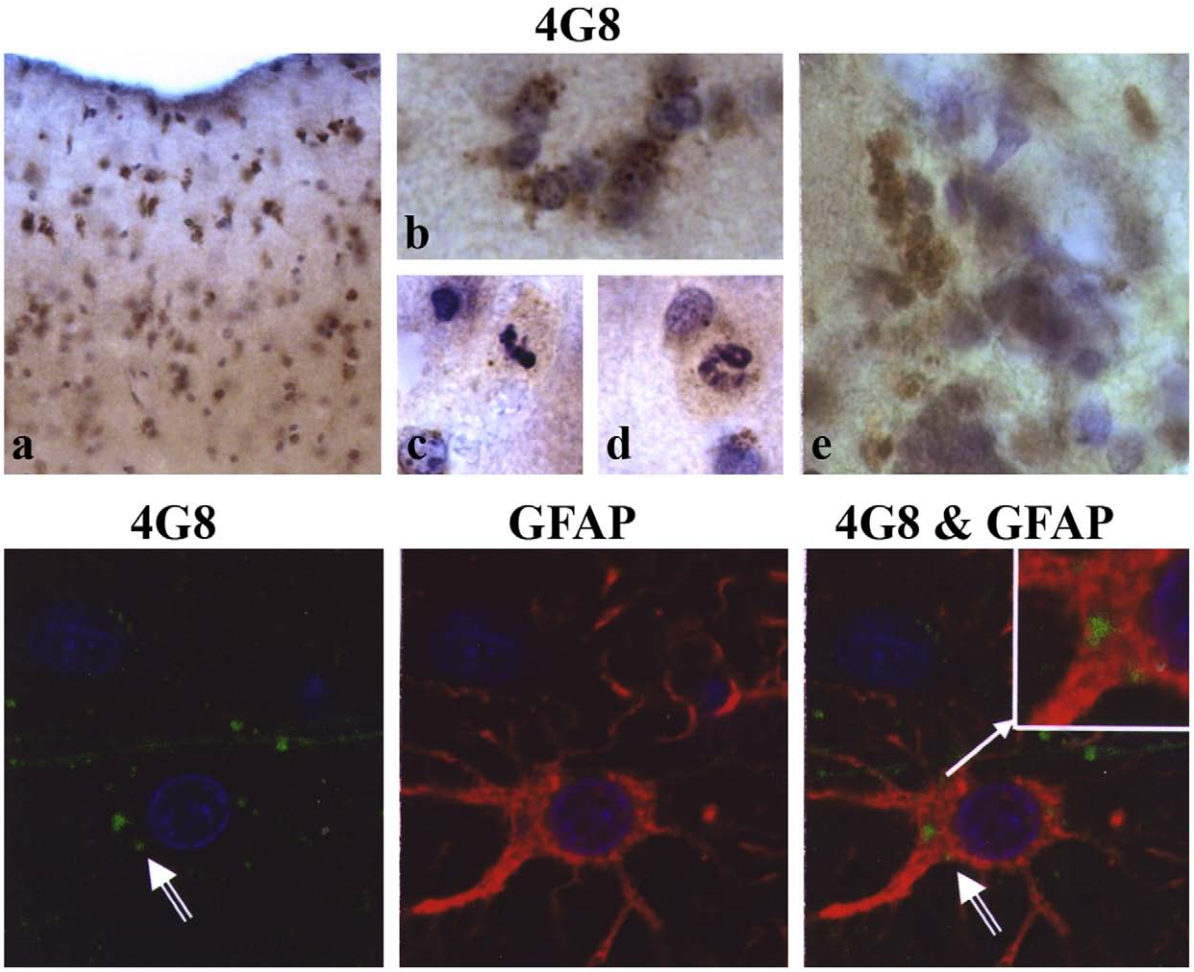

Figure 3. Enhanced accumulation of amino-terminally truncated Aβ in autistic subjects astrocytes. Clusters of 4G8-positive astrocytes, especially numerous in the molecular layer (a, b); very frequent mitotic divisions (c, d); and extracellular 4G8-positive Aβ deposits, with morphology of astrocytes' cytoplasmic aggregates (e) may reflect the enhanced proliferation, degeneration and death of Aβ-positive astrocytes in the brain of autistic subjects. Confocal microscopy confirmed the presence of Aβ (green; arrows) in the cytoplasm of GFAP-positive astrocytes (red). Cell nuclei were stained with TO-PRO-3-iodide (blue).
doi:10.1371/journal.pone.0035414.g003

distribution; however, they had the same immunoproperties. They revealed no reaction or traces of reaction with mAb 6E10 (Fig. 1) or 6F3D (not shown). The morphological diversity of Aβ deposits suggested that Aβ was present in different compartments of the endosomal-lysosomal pathway and in lipofuscin in neuron type–specific amounts. The number and size of Lamp1– (Fig. 4) lysosomes was from 2 to 3 times more than the number of Aβ-positive deposits; however, only about 10% of Aβ was detected in rab5-positive endosomal vesicles and in LC3B-positive autophagic vacuoles. Colocalization of Aβ with COXIV-positive mitochondria was observed in only a very few mitochondria.

Immunoreaction for Aβ detected with mAb 4G8 was present in some intracellular autofluorescent granules; however, the 4G8-immunoreactive deposits were detected also in neurons with scanty lipofuscin (Fig. 5) and in neurons with abundant autofluorescent granules. On the other hand, some neurons with scanty immunoreaction for Aβ contained numerous autofluorescent granules. The autofluorescent granules were not immunostained with mAb 6E10. Immunoreaction with polyclonal antibody (pAb) R226, specific for the C-terminus of Aβ42, showed only a fraction of labeling colocalized with autofluorescent granules. These results indicate that the detected intraneuronal immunostaining reflects accumulation of N-terminally truncated Aβ in several cellular compartments, including lipofuscin granules.

Specificity of Immunohistochemical Detection of Aβ with mAb 4G8 and 6E10

The epitopes of mAbs 6E10 and 4G8 (4–13 aa and 17–24 aa of the Aβ sequence, respectively) are present in full-length APP and APP C-terminal fragments. In brain tissue that has been fixed in formalin for several months, embedded in polyethylene glycol (PEG) and pretreated with 70% formic acid for 20 min, the immunostaining with mAb 4G8 (Fig. 6) and with 6E10 and 7F3D (8–17 aa of Aβ; not shown) is consistent with the distribution and amount of Aβ, but different from the distribution and amount of neuronal APP. In control brains, antibody R57 detects abundant intraneuronal APP immunoreactivity, but mAb 4G8 reveals only a very limited reaction with Aβ. In numerous neuronal populations in autistic subjects, the immunoreactivity for Aβ increases very significantly, but most R57 immunoreactive material is 4G8-negative, and most 4G8-positive granules are negative for APP. These results indicate that in the examined material, mAbs 6E10,

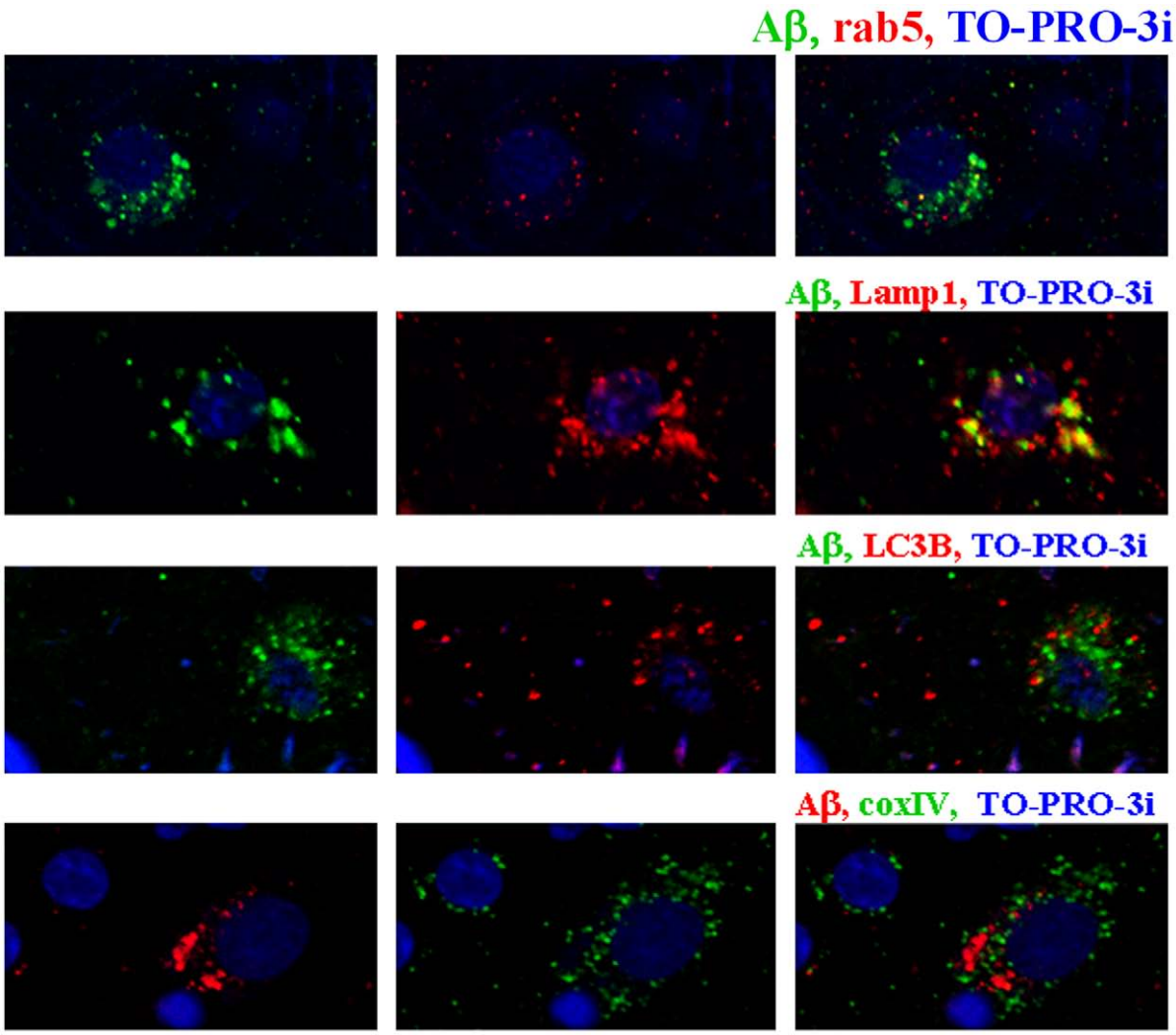

Figure 4. Aβ in endocytic vesicles, autophagic vacuoles, lysosomes and mitochondria. Co-localization of Aβ (4G8) in neurons in the frontal cortex of a 10-year-old subject diagnosed with autism/dup(15) (AN06365) demonstrates that a small portion of cytoplasmic Aβ is stored in rab5-positive endocytic vesicles and LC3B-positive autophagic vacuoles, whereas the largest proportion of Aβ is colocalized with lysosomal Lamp1. Colocalization of a relatively large portion of cytoplasmic Aβ with lysosomal markers appears to reflect the accumulation of products of intracellular degradation of Aβ that originated from endocytic and autophagic pathways. The presence of only a few Aβ-positive mitochondria immunolabeled with COXIV may suggest that this Aβ makes the smallest contribution to the detected neuronal Aβ accumulation and degradation pathway.
doi:10.1371/journal.pone.0035414.g004

4G8 and 7F3D detect Aβ but do not bind to neuronal APP detected with pAb R57.

Diffuse Plaque Distribution and Immunoproperties in the Brain of Autistic Subjects

Aβ-positive plaques were detected in one of the nine examined subjects diagnosed with dup15 (AN11931), and in two of the 11 subjects diagnosed with idiopathic autism (AN17254 and BB1376). All three subjects were the oldest in each group. In the dup(15) group, a 39-year-old female with autistic features and intractable epilepsy (onset at 9 years of age) and whose death was epilepsy-related had clusters of plaques in several neocortical regions, including the frontal, temporal and insular cortex (Fig. 7). Plaques were also found in the brains of two individuals diagnosed with idiopathic autism, including a 51-year-old subject who had had only one grand mal seizure (Fig. 8), and a 52-year-old individual whose records do not contain information about epilepsy or brain trauma. In both brains, the postmortem examination revealed numerous plaques within the entire cortical ribbon (Fig. S2) and in the amygdala, thalamus and subiculum (not shown).

In all three cases, thioflavin S staining did not reveal fluorescence in the plaques (not shown), suggesting that the amyloid plaques detected in the examined subjects with autism/dup(15) and idiopathic autism were nonfibrillar. However, positive immunoreactivity with all six antibodies used, including 6E10, 6F3, 4G8, Rabm38, Rabm40 and Rabm42 (Fig. 7 and 8) and 6F3D (not shown), revealed full-length $A\beta_{1-40/42}$ peptides. In the plaque area, numerous glial cells, mainly with the morphology of astrocytes, and less numerous, glial cells with the morphology of microglial cells, contained Aβ-immunoreactive granular material. In contrast to the presence of full-length Aβ peptides in plaques, the Aβ peptides in both astrocytes and microglial cells in the plaque perimeter and surrounding tissue were mAb 6E10- and 6F3D-negative, indicating that they were the product of α-secretase. They were positive for the three other antibodies,

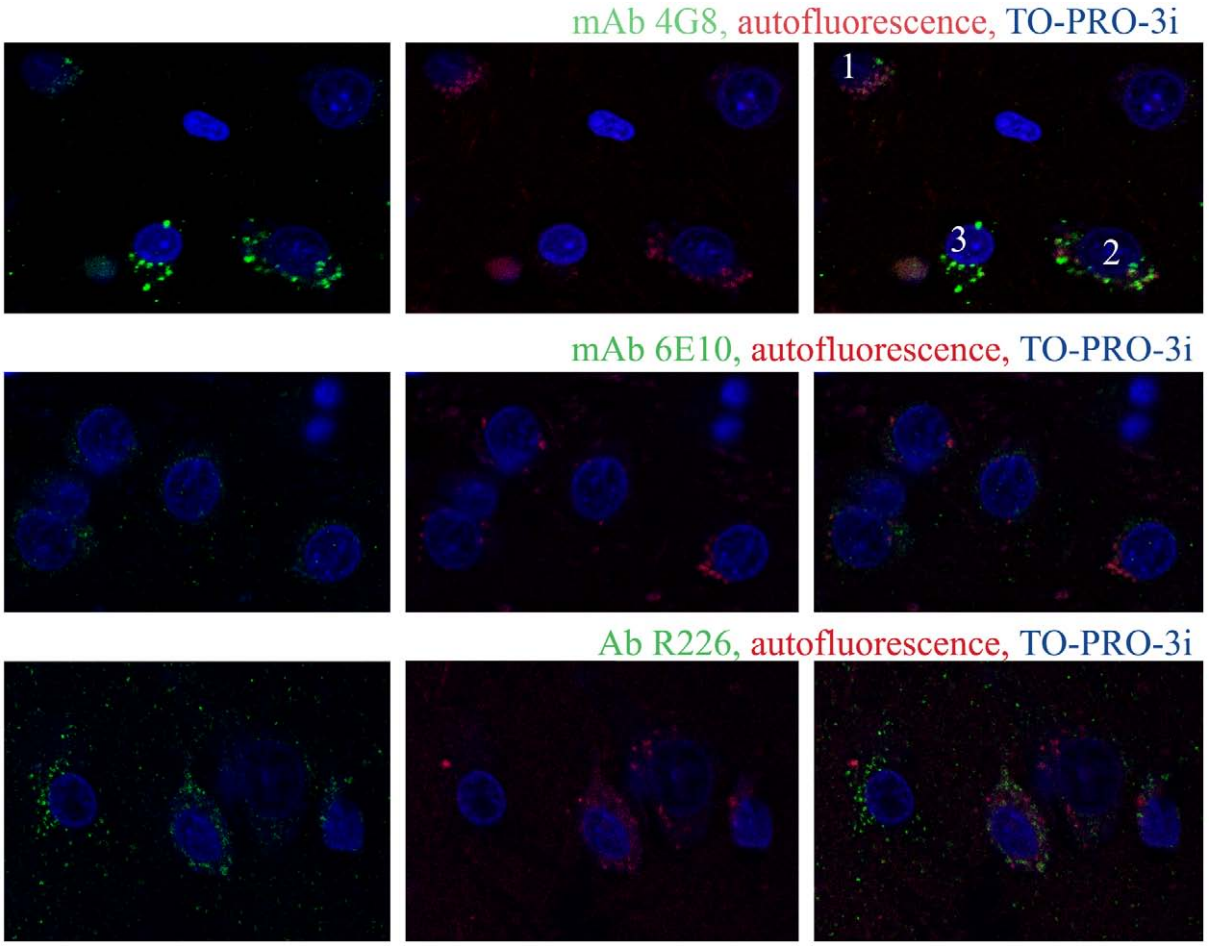

Figure 5. Aβ in lipofuscin. Accumulation of Aβ in lipofuscin in the frontal cortex of a 10-year-old subject diagnosed with dup(15) autism was characterized using mAbs 4G8 and 6E10 and pAb R226. Autofluorescent lipofuscin granules were 4G8-negative (cell 1) or partially positive (cell 2), but Aβ was also accumulated in lipofuscin-free neurons (cell 3). The neurons revealed only traces of reaction with mAb 6E10 and a moderate amount of pAb R226–positive Aβ42, which was partially co-localized with autofluorescent lipofuscin.
doi:10.1371/journal.pone.0035414.g005

Rabm38, Rabm40 and Rabm42, demonstrating that both astrocytes and microglia accumulate $A\beta_{17–40/42}$.

The extracts from the areas of the cerebral cortex in which diffuse plaques were detected by immunohistochemistry contained Aβ, mainly Aβ1–42, revealed by immunoblotting as a 4-kD band reacting with pAb R226 and mAb 6E10. The levels of Aβ1–42 in the samples exceeded 1.5 fmol per 1 μg of extracted proteins, whereas the levels of extracted Aβ 1–40 were low, below 0.2 fmol per 1 μg of extracted proteins (Fig. 9).

Immunoblotting of lysates from the cerebral cortex of autistic subjects without plaques and age-matched control subjects detected Aβ42 (Fig. 10) and Aβ40 (not shown) as a 3- to 4-kD band reacting with the pAb R226 and pAb R162, respectively. The levels of Aβ42 in the samples were in the range below 0.5 fmol per 40 μg of total proteins.

Neurofibrillary Degeneration

A very few neurofibrillary tangles (NFTs) were found in the entorhinal cortex and amygdala in a 43-year-old control subject and in the entorhinal cortex and cornu Ammonis of a 47-year-old control subject. A few NFTs were found in the entorhinal cortex, CA1 and parasubiculum in a 51-year-old autistic subject and in the entorhinal and temporal cortex and the amygdala of a 52-year-old autistic subject. Neurofibrillary changes were not found in the dup(15) autism cohort with the oldest examined subject who died at the age of 39 years.

Discussion

The accumulation of intraneuronal Aβ is considered a first step leading to amyloid plaque formation in AD [14,22–24]. However, our examination of control brains during the life span showed that intraneuronal Aβ also occurs in normal controls and that almost all cytoplasmic Aβ peptides are the product of α- and γ-secretases ($A\beta_{17–40/42}$) [21], whereas, the majority of amyloid in plaques is the product of β- and γ-secretases. This finding suggests that brain region– and neuron type–specific patterns of intraneuronal $A\beta_{17–40/42}$ peptide accumulation in control brains are a baseline for detection and evaluation of increases associated with autism, FXS, epilepsy, brain trauma or age-associated neurodegeneration, such as AD.

Detection of Aβ in Human Postmortem Material

The epitopes of mAbs 6E10 (4–13 aa of the Aβ sequence) and 4G8 (17–24 aa) are present in full-length APP and various APP fragments. Recently, Winton et al. [25] demonstrated that neuronal APP is immunolabelled with these two antibodies in

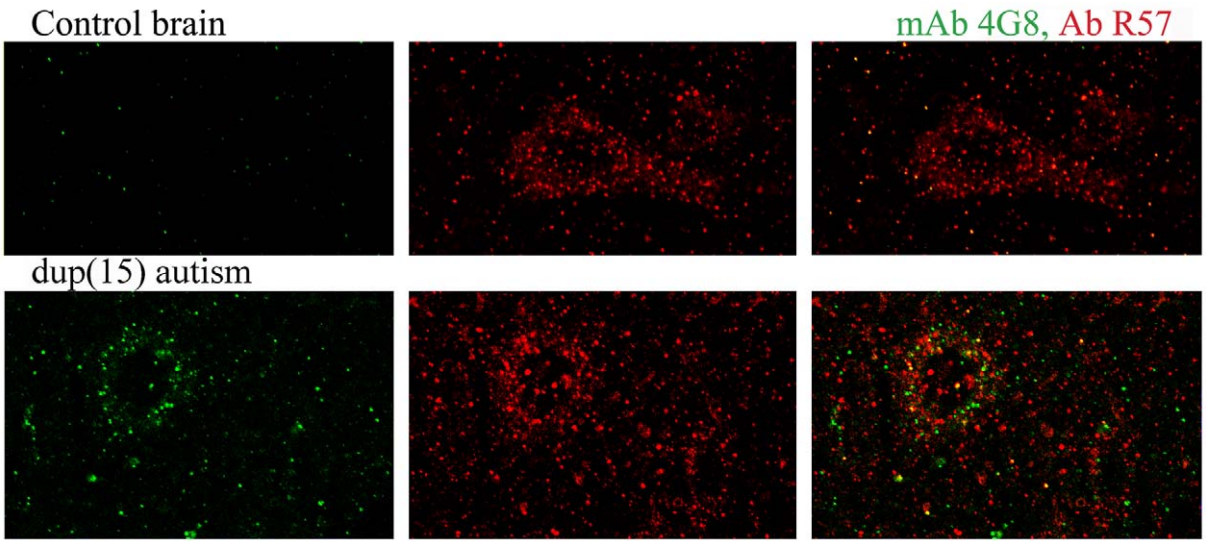

Figure 6. Immunoreactivity of mAb 4G8 with Aβ. mAb4G8 detects Aβ but does not detect APP in immunohistochemical staining in formalin-fixed and PEG-embedded samples of the frontal cortex of an 8-year-old control subject and a 10-year-old subject diagnosed with dup(15) and autism. Neurons in the control brain contain numerous granules that are immunoreactive with C-terminal APP–specific pAb R57 and are 4G8 negative. In the neurons of an autistic subject, only a few very numerous 4G8-positive deposits are R57-positive, whereas the majority of very numerous APP-immunoreactive granules are 4G8-negative.
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mouse brain fixed for 24 hours in 10% neutral buffered formalin. However, the pattern of immunostaining in human brain fixed in formalin for at least several months, dehydrated almost 3 weeks in ascending concentrations of ETOH, and embedded in PEG indicates that mAbs 4G8, 6E10 and 6F3D do not detect APP in tissue subjected to this process. The role of technical factors in the loss of access of these antibodies to their epitopes in APP was previously documented in studies of tissue fixed in formalin for 10 days and in studies of cultured cells [26,27]. Several observations in this report indicate that these antibodies do not detect APP. Massive immunolabelling of neuronal APP with R57 is in striking contrast with the presence of only traces of 6E10 and 6F3D immunoreactivity in these cells and the only partial co-localization of Aβ and APP labeling in the amyloid-rich neurons of autistic subjects. These data indicate that in the examined material, APP is detected with the APP-specific antibody R57, but not with mAbs 4G8, 6E10,and 6F3D, which, however, detect Aβ. One may assume that the epitopes of these antibodies, but not the R57 epitopes, are blocked or modified in APP molecules during long exposure to chemicals used for fixation, dehydration and embedding. Consistent with immunocytochemistry, Western blotting identifies 3–4 kD Aβ not only in subjects with diffuse plaques, but also in autistic subjects without plaques and in control subjects.

Excessive Accumulation of $A\beta_{17-24}$ in Neurons in Idiopathic Autism and dup(15) Autism

This is the first report documenting excessive accumulation of Aβ in the neurons of subjects with idiopathic autism and an even more pronounced accumulation in the dup(15) autism cohort. Two patterns of excessive accumulation distinguish these two cohorts from control subjects and indicate that excessive accumulation is neuron type/brain region–specific. Type 1 of altered Aβ accumulation is reflected in an increase in the percentage of neurons with strong Aβ accumulation by 7.6-fold in the amygdala and thalamus and by 4.5-fold in the LGB in individuals with dup(15) autism in comparison to the control group. A similar (by 5.3×, 6.3× and 3.9×, respectively) and statistically significant increase was found in the idiopathic autism group. Type 2 of altered Aβ accumulation is reflected in a more uniform increase in the percentage of neurons with combined strong, moderate and weak immunoreactivity. Again, this pattern is observed in both autistic cohorts in the pyramidal neurons in all three examined cortical regions.

These findings suggest that metabolic alterations are similar in both types of autism and that the severity of these alterations is less pronounced in idiopathic autism than in autism caused by dup(15). The significant increase in the percentage of neurons with enhanced cytoplasmic Aβ load in idiopathic autism and the fact that almost all of this Aβ is the product of α-secretase show the striking similarity to increased levels of sAPP-α in blood plasma in 60% of autistic children (6,16). In studies by Sokol et al. [6] and Ray et al. [16], aggressive behavior was identified as associated with increased levels of sAPP-α. Bailey et al. [9] also detected a significant increase in sAPP-α levels in 60% of autistic children but with no association between the severity of aggression, social or communication sub-scores and increased levels of sAPP-α. Due to the neurotrophic properties of sAPP-α, the authors proposed that an increased level of the products of α-secretase may help identify a subset of children in which early regional brain overgrowth is necessary and sufficient for the development of autism and may even represent a mechanism regulating overgrowth in autism. However, the most pronounced accumulation of amino-terminally truncated Aβ observed in the dup(15) autism cohort with microcephaly [28] indicates that intraneuronal Aβ accumulation of the products of α-secretase is not associated with brain overgrowth. Our data identify a dup(15) autism subcohort with microcephaly, more severe clinical phenotype, very early onset of seizures, a high percentage of intractable seizures, and a high prevalence of sudden unexpected death in epilepsy (SUDEP) as associated with the highest percentage of neurons accumulating α-secretase product.

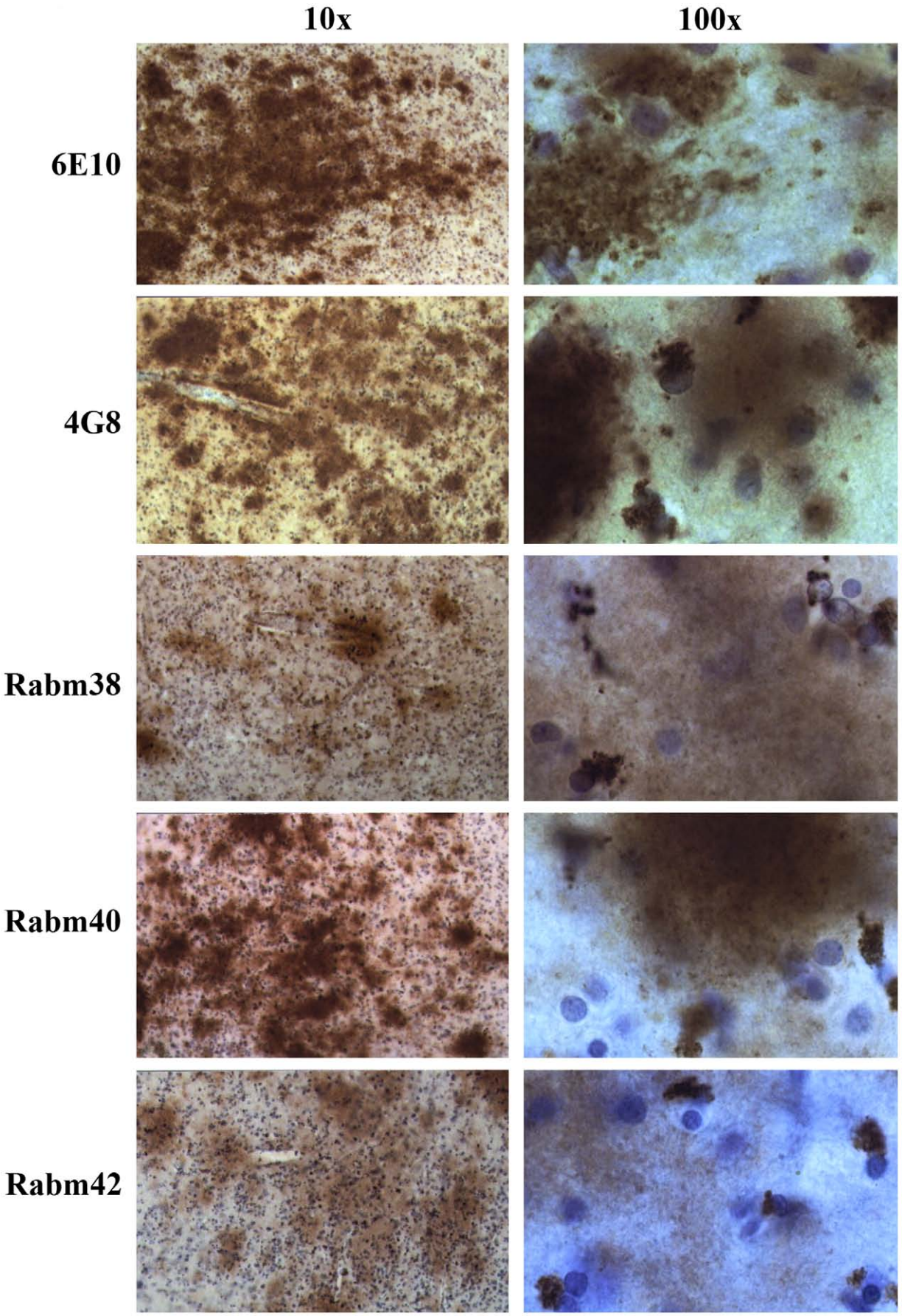
10x
100x
6E10
4G8
Rabm38
Rabm40
Rabm42

Figure 7. Full-length Aβ in diffuse plaques and amino-terminally truncated Aβ in astrocytes in autism/dup15. Diffuse plaques in the frontal cortex of a 39-year-old female (AN11931) diagnosed with dup(15), autistic features, and intractable seizures (age of onset 9 years) and whose death was epilepsy-related, are 6E10-, 4G8-, Rabm38-, Rabm40- and Rabm42-positive. Reaction with Rabm38 and Rabm42 was weaker than with other antibodies. Almost all glial cells with the morphology of astrocytes detected in the plaque perimeter had a large cluster of granular material located usually at one cell pole and positive with all antibodies detecting Aβ, except 6E10.
doi:10.1371/journal.pone.0035414.g007

Trafficking of $A\beta_{17–24}$ in Neurons

Aβ is generated in the endolysosomal pathway and in the endoplasmic reticulum/Golgi compartment [29–33] and is also detected in multivesicular bodies [34] and in mitochondria [15,35]. The application of Lamp1 as a lysosomal marker revealed that approximately 20–30% of neuron cytoplasmic $A\beta_{17–24}$ accumulates in this step of the proteolytic pathway in control and autistic subjects. An increase in cathepsin D protein expression, as reported in several brain regions of autistic subjects, suggests the selective enhancement of target proteins' hydrolysis by this aspartic acid protease [36]. The lysosome is the major acid hydroxylase-containing cell compartment engaged in processing of substrates delivered by (a) endocytosis, (b) autophagy [37] and (c) scavenging of proteins from the endoplasmic reticulum to lysosomes [38]. The increase of $A\beta_{17–24}$ in the lysosomes of autistic subjects may reflect $A\beta_{17–40/42}$ generation in these pathways.

This study revealed that another 20–30% of neuron $A\beta_{17–40/42}$ is present in lipofuscin, which is the final product of cytoplasmic proteolytic degradation of exogenous and endogenous substrates. During the entire lifespan, lipofuscin gradually accumulates in neurons [39]. The age of onset and dynamics of lipofuscin deposition are cell type–specific [40,41]. Our previous study revealed that neurons in the inferior olive, dentate nucleus and lateral geniculate body start accumulating lipofuscin and $A\beta_{17–40/42}$ early in life and that this accumulation progresses with age at region-specific rates [21]. The confocal microscopy study indicates that in spite of the known nonspecific binding of some antibodies to lipofuscin, the selection of the immunostaining protocol and the setting of proper thresholds in confocal imaging applied in this study reveal the selectivity of mAbs 4G8 and 6E10, and pAb R226 binding to some lipofuscin deposits.

The pattern of both Aβ and lipofuscin accumulation can be modified in early childhood in subjects with autism and even more significantly in individuals with dup(15) autism. The difference is detectable as an increase in the percentage of $A\beta_{17–40/42}$ immunoreactive neurons, the amount of immunopositive material per neuron, and the number of brain regions and neuron types affected in both children and adults. Detected changes in Aβ accumulation may reflect abnormal accumulation of lipofuscin, as reported by Lopez-Hurtado and Prieto [42]. An increase in the number of lipofuscin-containing neurons by 69% in Brodmann area (BA) 22, by 149% in BA 39, and by 45% in BA 44, in brain tissue samples from autistic individuals 7 to 14 years of age, was observed together with a loss of neurons and glial proliferation. However, enhanced lipofuscin accumulation is not unique for idiopathic autism or autism/dup(15). It has been reported in Rett syndrome [43], an ASD, as well as in several psychiatric disorders, including bipolar affective disorder [44] and schizophrenia [45,46].

Enhanced lipofuscin accumulation and enhanced $A\beta_{17–40/42}$ immunoreactivity in the majority of the examined brain structures in most of the individuals with autism and the subjects with dup(15) may be a reflection of enhanced oxidative stress. Oxidative stress contributes to protein and lipid damage in cytoplasmic components, their degradation in lysosomal and autosomal pathways, and the deposition of products of degradation in lipofuscin or their exocytosis [47,48]. The link between oxidative stress, cytoplasmic degradation and lipofuscin deposition is supported by the presence of oxidatively modified proteins and lipids in lipofuscin [39,49,50]. A significant increase in malondialdehyde levels (a marker of lipid peroxidation) in the plasma of autistic children [51] and in the cerebral cortex and cerebellum [52] may reflect oxidative damage leading to enhanced degradation, and the possible increased turnover of affected cell components.

Biological Activity of N-terminally Truncated Aβ

The results of confocal microscopy suggest that on average, 30% of neuronal Aβ is present in lysosomes and another 30% in lipofuscin. However, the biological consequences of accumulation of Aβ, in the lysosomes or in lipofuscin are not known. N-terminally truncated Aβ peptides exhibit enhanced peptide aggregation relative to the full-length species [53] and retain their neurotoxicity and β-sheet structure. Soluble intracellular oligomeric Aβ (oAβ) species inhibit fast axonal transport (FAT) in both anterograde and retrograde directions [54]. Inhibition of FAT results from activation of endogenous casein kinase 2. Altered regulation of FAT markedly reduces transport of synaptic proteins and mitochondria in the AD brain and in AD mouse models that accumulate oAβ [55]. Dysregulation of FAT results in distal axonopathies with a reduced delivery of critical synaptic elements required for the integrity, maintenance and function of synapses [54].

The *in vitro* studies suggest that Aβ 17–24 is toxic to neurons. Treatment of SH-SY5Y and IMR-32 human neuroblastoma cells with Aβ 17–24 causes apoptotic death similar to in cells incubated with Aβ1–42, whereas treatment with Aβ17–40 results in a lower level of apoptosis, comparable to experimental exposure to Aβ1–40. This apoptosis is mediated predominantly by the caspase-8 and caspase-3 pathways [56]. However, *in vitro* studies of the neuronal response to exogenous Aβ peptides do not replicate the neuronal exposure to endogenous $A\beta_{17–40/42}$ trafficking inside vesicles and vacuoles of lysosomal pathway.

$A\beta_{1–40/42}$ in Diffuse Plaques of Autistic Subjects

The presence of diffuse nonfibrillar plaques in two autistic subjects who were more than 50 years old and in one 39-year-old subject with autism/dup(15) suggests that in the fourth/fifth decade of life, there is an increased risk of the second type of changes: activation of the amyloidogenic pathway of APP processing with β- and γ-secretases, resulting in focal deposition of $A\beta_{1–40/42}$ in plaques. It was hypothesized that $A\beta_{17–42}$ peptides may initiate and/or accelerate plaque formation, perhaps by acting as nucleation centers that seed the subsequent deposition of relatively less amyloidogenic but apparently more abundant full-length Aβ [53,57,58]. Gouras et al. [59] considered intracellular $A\beta_{42}$ accumulation an early event leading to neuronal dysfunction. The $A\beta_{1–40/42}$ –positive diffuse plaques in the brains of autistic subjects are different from the $A\beta_{17–40/42}$–positive cerebellar diffuse plaques detected in DS [57,60]. Diffuse amorphous nonfibrillar Aβ deposits, called amorphous plaques [61], pre-plaques [62] or pre-amyloid deposits [63], are considered to be of neuronal origin [64–67]

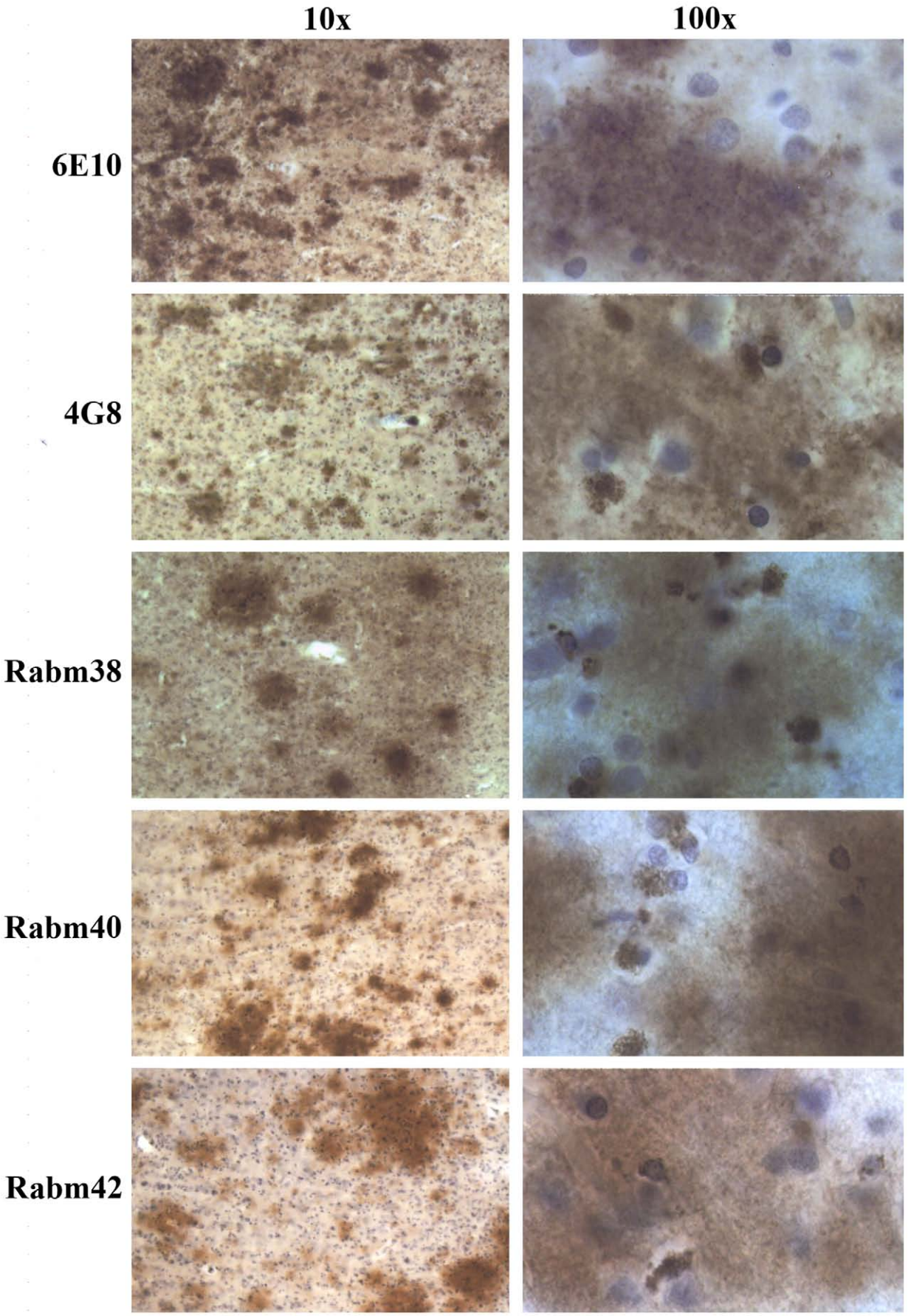
10x
100x
6E10
4G8
Rabm38
Rabm40
Rabm42

Figure 8. Full-length Aβ in diffuse plaques, and truncated Aβ in astrocytes in idiopathic autism. Diffuse plaques in the frontal cortex of a 51-year-old subject (AN17254) diagnosed with idiopathic autism, who had had only one grand mal seizure and died because of cardiac arrest, are immunopositive when stained with all five antibodies (6E10, 4G8, Rabm38, Rabm 40 and Rabm 42), but granular material in the cytoplasm of glial cells is immunopositive for all antibodies used except 6E10.
doi:10.1371/journal.pone.0035414.g008

and are formed selectively in projection areas of distant affected neuronal populations [68]. Diffuse plaque formation in autistic subjects suggests the activation of the secretory pathway and the synaptic release of $A\beta_{1-40/42}$.

The presence of $A\beta_{17-40/42}$ in astrocytes in $A\beta_{1-40/42}$ –positive diffuse plaques suggests that the full-length Aβ released by neurons is phagocytosed and processed by local astrocytes. One may hypothesize that the proliferation of Aβ-positive astrocytes, the increase of cytoplasmic Aβ immunorectivity in astrocytes, the presence of Aβ in all astrocytes in the affected region, astrocyte death and the deposition of large aggregates of extracellular Aβ in the cerebral cortex or hippocampus of autistic children and young adults is a response to the elevated levels of extracellular $A\beta_{17-40/42}$ and/or $A\beta_{1-40/42}$. Therefore, the number of Aβ-positive astrocytes may be an indicator of the local concentration of extracellular Aβ not only in plaque-positive but also in plaque-negative brain regions, occurring decades before plaque formation. Cytoplasmic granular immunoreactivity (Aβ17–23 and Aβ8–17) was reported in astrocytes in AD [69]. In astrocytes, intracellular Aβ appears in lysosomes and lipofuscin [70,71]. It defines the role of astrocytes in the uptake of different species of Aβ in diffuse and neuritic plaques and their subsequent degradation in lysosomes and storage of products of degradation in lipofuscin [69].

In the examined autistic cohort, the early onset of intractable epilepsy and the epilepsy-related chronic and acute brain trauma appear to be additional risk factors for APP pathway activation and diffuse plaques formation. Repetitive brain trauma, including that related to epilepsy and head banging, produces a chronic traumatic encephalopathy with the associated deposition of Aβ, most commonly as diffuse plaques [72–74]. In acute traumatic brain injury, diffuse cortical Aβ deposits were detected in 30% to 38% of cases 2 hours after injury [75–77].

The presence of a few NFTs in the entorhinal cortex, cornu Ammonis and amygdala in 43- and 47-year-old control subjects and in these structures and in the parasubiculum and temporal cortex of 51- and 52-year-old autistic subjects is consistent with the topography and amount of age-associated neurofibrillary degeneration and NFT distribution observed in the general population [78].

In conclusion, this postmortem study of Aβ distribution in the brain of subjects with idiopathic autism and dup(15) autism suggests (a) very significant enhancement of intraneuronal Aβ accumulation in almost all examined cortical and subcortical structures in autism, especially in autism associated with dup(15); (b) the prevalence of anabolic α-secretase APP processing and $A\beta_{17-40/42}$ accumulation in neuronal endosomes, autophagic vacuoles, lysosomes and lipofuscin in the majority of autistic children and adults; and (c) activation of the amyloidogenic pathway of APP processing with β- and γ-secretases in the late adulthood of some autistic subjects with diffuse nonfibrillar plaque formation and astrocyte activation.

Materials and Methods

Material, Clinical and Genetic Evaluation

The brains studied were from nine individuals diagnosed with dup(15) ages 9 to 39 years (five males and four females), 11 subjects with idiopathic autism ages 2 to 52 years (10 males and one female), and eight control subjects ages 8 to 47 years (four males and four males) (Table 1). Medical records were obtained following consent for release of information from the subjects' legal

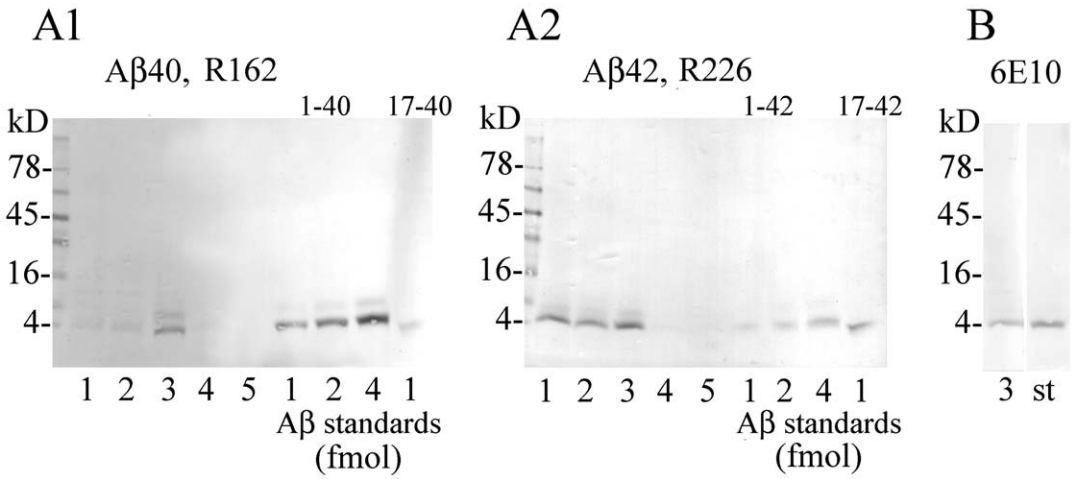

Figure 9. Properties of Aβ in plaque-rich cortex characterized by Western blotting. Panels A1 and A2 show Aβ40 and Aβ42 detected with pAbs R162 and R226, respectively, in blots of extracts (3 μg of total proteins per line) from cerebral cortex containing diffuse plaques of a 39-year-old subject with dup(15) (lane 1), of 51- and 52-year-old individuals with idiopathic autism (lanes 2 and 3), and of 48- and 47-year-old controls (lane 4 and 5). Blots reveal full-length Aβ, mainly Aβ42, in samples from plaque-positive subjects but not in controls. As standards, 1, 2 and 4 fmols of synthetic Aβ1–40, 17–40 (panel A1) and Aβ1–42, 17–42 (panel A2) were used. Panel B shows Aβ detected with mAb 6E10 specific for the N-terminal portion of Aβ in extract from the cortex of the 52-year-old subject (lane 3; 6 μg of protein per lane) and 4 fmol of synthetic Aβ1–40 (st). Panels A1, A2 and B demonstrate that in the extracts from diffuse plaque–positive cortical samples of autistic subjects, the levels of Aβ1–40 and 1–42 exceeded 1.5 fmol per 1 μg of extracted protein.
doi:10.1371/journal.pone.0035414.g009

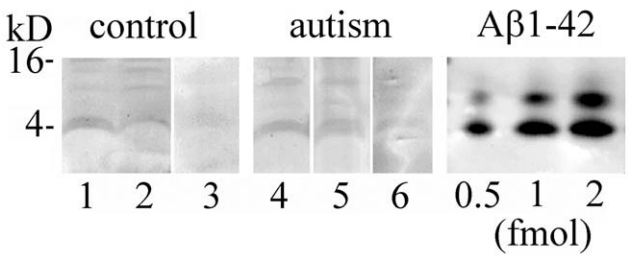

Figure 10. Detection by Western blots of Aβ in plaque-free subjects. Aβ42 detected with pAb R266 in lysates from cerebral cortex of control individuals 31, 32 and 51 years old (lanes 1–3, respectively), and individuals with idiopathic autism 8, 22, 29, and 29 years old (lane 4, 6, respectively). 40 μg of total lysate proteins were loaded per lane. Synthetic Aβ1–42 was used as a standard.
doi:10.1371/journal.pone.0035414.g010

guardians. The study was approved by the Institutional Review Boards for the New York State Institute for Basic Research in Developmental Disabilities; the University of California, Los Angeles; and Nemours Biomedical Research, duPont Hospital for Children, Wilmington. Clinical and genetic studies were performed as described previously [28]. Clinical characteristics were based on psychological, behavioral, neurological and psychiatric evaluation reports. To confirm a clinical diagnosis of autism, the Autism Diagnostic Interview-Revised (ADI-R) was administered to the donor family [79].

Molecular genetic evaluations, using antemortem peripheral blood samples and lymphoblast cell lines for eight of the dup(15) cases, included genotyping with 19–33 short tandem repeat polymorphisms from chromosome 15, Southern blot analysis of dosage with 5–12 probes, measurement of the methylation state at *SNRPN exon* α, as described [80], and array comparative genomic hybridization [81]. Duplication morphology was confirmed by fluorescent in situ hybridization [80].

In eight cases, tetrasomy, and in one case, hexasomy of the Prader-Willi/Angelman syndrome critical regions was detected. In eight cases, the origin of abnormality was maternal; in one case, the origin was not determined. In the examined dup(15) group, seven of nine subjects (78%) were diagnosed with autism or ASD, and seven had seizures. In six cases (67%), SUDEP was reported. In the idiopathic autism cohort, two subjects (8-year-old male, HSB4640, and 52-year-old male, BB1376), were diagnosed with the ASD (pervasive developmental disorder – not otherwise specified and high-functioning atypical autism, respectively). In all other cases, the clinical diagnosis of autism was confirmed with ADI-R.

One brain hemisphere was preserved for neuropathological and immunocytochemical studies. Methods and results of neuropathological evaluations of developmental abnormalities have been summarized in our previous reports [28,82]. The mean postmortem interval varied from 23.9 h in the dup(15) cohort to 19.6 h in the idiopathic autism cohort and 15.0 h in the control group. One brain hemisphere from each subject was fixed in 10% buffered formalin for a period ranging from six weeks to several months, dehydrated in a graded series of ethanol, infiltrated and embedded with PEG (Sigma) [83] and stored at 4°C. Tissue blocks were then cut into 50-μm-thick serial sections and stored in 70% ethyl alcohol. Two brains (AN17254 and BB1376) were embedded in celloidin, as described [82] and were cut alternatively into 200- and 50-μm-thick serial sections.

Immunocytochemistry and Confocal Microscopy

Brain Bank identification of the tissue samples is listed in Table 1, to maintain non-overlapping records of results of brains examined in different projects. Immunocytochemistry and confocal microscopy were applied to characterize (a) Aβ distribution in cells in the cerebral cortex, subcortical structures, cerebellum and brainstem and in diffuse plaques; (b) the Aβ peptide properties; and (c) Aβ distribution in endosomes, lysosomes, autophagic vacuoles, mitochondria and lipofuscin (Table 2).

mAbs 6E10 (Covance, Inc., Princeton, Inc.) and 6F3D (Novocastra Lab. Ltd., Newcastle, UK) were used to characterize the N-terminal portion of Aβ. mAb 6E10 recognizes an epitope in residues 4–13 of Aβ [84,85]. mAb 6F/3D recognizes an epitope in residues 8–17 of Aβ. The middle portion of Aβ was detected with mAb 4G8, which recognizes an epitope in residues 17–24 of Aβ [86]. The carboxyl terminus of Aβ was characterized with rabbit monoclonal antibodies Rabm38, Rabm40 and Rabm42, which detect $A\beta_{-38}$, $A\beta_{-40}$, and $A\beta_{-42}$, respectively [87]. The specificity of mAbs 4G8 and 6E10 for Aβ was verified in the examined postmortem human brain tissue by double immunolabeling with pAb R57 detecting APP C-terminal aa 671–695.

To detect intracellular Aβ peptides and amyloid in plaques, free-floating sections were treated with 70% formic acid for 20 minutes [88]. The endogenous peroxidases in the sections were blocked with 0.2% hydrogen peroxide in methanol. The sections were then treated with 10% fetal bovine serum in phosphate buffer solution (PBS) for 30 minutes to block nonspecific binding. The primary antibodies were diluted in 10% fetal bovine serum in PBS and sections were treated overnight at 4°C. The sections were washed and treated for 30 min with either biotinylated sheep anti-mouse IgG antibody or biotinylated donkey anti-rabbit IgG antibody diluted 1:200. The sections were treated with an extravidin peroxidase conjugate (1:200) for 1 h, and the product of reaction was visualized with diaminobenzidine (0.5 mg/mL with 1.5% hydrogen peroxide in PBS). After immunostaining, sections were lightly counterstained with cresyl violet. To detect fibrillar Aβ in plaques, sections were stained with Thioflavin S and examined in fluorescence.

Neurons with fibrillary tangles were immunolabelled with mAb Tau-1, detecting an epitope between amino acids 189 and 207 of the human tau protein sequence [89]. To detect abnormally phosphorylated tau with Tau-1, sections were pretreated with alkaline phosphatase (Sigma, Saint Louis, MO; Type VII-L, 400 μg/ml in PBS, pH 7.4, 0.01% H_20_2).

Double immunofluorescence for Aβ (mAb4G8) and for astrocytes (GFAP; rabbit polyclonal antibody, pAb, Sigma) was carried out to confirm the presence of Aβ in astrocytes. Confocal microscopy was applied to detect Aβ localized in neuronal cytoplasmic organelles. Aβ in lysosomes was detected by using lysosomal-associated membrane protein marker (LAMP1; Abgent, San Diego, CA). Early endosomes were immunodetected with rabbit pAb Rab5 (Ab13253; Abcam, Cambridge, MA), whereas autophagic vacuoles were immunolabelled with rabbit mAb LC3B (Cell Signaling Technology Inc., Danvers, MA). Mitochondria were detected with the rabbit mAb COXIV Alexa Fluor 488 conjugated (Cell Signaling Technology). To detect Aβ, brain sections were treated with 70% formic acid for 20 min, washed in PBS 2x 10 min and double- immunostained using mAb 4G8 and antibodies detecting markers of cytoplasmic organelles. Affinity-purified donkey antisera against mouse IgG labeled with Alexa Fluor 488 and against rabbit IgG labeled with Alexa Fluor 555 (both from Molecular Probes/Invitrogen) were used as secondary antibodies. TO-PRO-3-iodide (Molecular Probes/Invitrogen) was used to counterstain cell nuclei. Absence of cross-reaction was

Table 1. Material examined, cause of death, and the prevalence of epilepsy.

Group	Brain Bank number	Sex	Age (y)	Cause of death	Epilepsy. age of onset
dup(15)	AN14762	M	9	SUDEP	IE/10 m
dup(15)	AN06365	M	10	SUDEP	IE/8 m
dup(15)	AN09402	M	11	SUDEP	IE/10 m
dup(15)	AN07740	F	15	SUDEP	E/11 y
dup(15)	AN09470	F	15	Aspiration pneumonitis	–
dup(15)	AN03935	M	20	Cardiopulmonary arrest	–
dup(15)	AN05983	M	24	Pneumonia	IE/7 y
dup(15)	AN14829	F	26	SUDEP	E/16 y
dup(15)	AN11931	F	39	SUDEP	IE/9 y
Autism	AN03345	M	2	Asphyxia (drowning)	–
Autism	AN13872	F	5	Asphyxia (drowning)	–
Autism	AN08873	M	5	Asphyxia (drowning)	–
Autism	HSB4640	M	8	Asthma attack	E/8 y
Autism	AN01293	M	9	Heart failure	–
Autism	CAL105	M	11	Asphyxia (drowning)	E
Autism	IBR93-01	M	23	Seizure related	E/23 y
Autism	AN08166	M	28	Seizure-related	E
Autism	NP06-54	M	32	Brain tumor	–
Autism	AN17254	M	51	Heart failure	1 grand mal
Autism	BB1376	M	52	Heart failure	–
Control	UMB1706	F	8	Rejection of cardiac transplant	–
Control	UMB1670	M	14	Asphyxia (hanging)	–
Control	UMB4722	M	14	Multiple traumatic injuries	–
Control	BTB3960	F	25	Not known	–
Control	IBR291-00	M	32	Heart failure	–
Control	IBR212-98	F	33	Bronchopneumonia	–
Control	IBR38-98	F	43	Sepsis	–
Control	IBR457-96	M	47	Myocardial infarct	–

Sudden unexpected and unexplained death of subject with known epilepsy (SUDEP), Intractable epilepsy (IE), Epilepsy (E), Years (y), Months (m).
doi:10.1371/journal.pone.0035414.t001

confirmed as previously described [26]. Images were generated using a Nikon C1 confocal microscope system with EZC1 image analysis software.

Comparison of Intraneuronal Aβ Accumulation in Examined Cohorts

Semiquantitative estimation of intraneuronal Aβ was performed without knowledge of the subject's age, gender or clinical diagnosis or the neuropathological diagnosis of the tissue being analyzed. Evaluation was performed at a workstation consisting of Axiophot II light microscope, specimen stage with 3-axis computer-controlled stepping motor system (Ludl Electronics; Hawthorne, NY), CCD color video camera (CX9000 MicroBrightField Bioscience, Inc., Williston, VT) and stereology software (Stereo Investigator, MicroBrightField Bioscience Inc.). Grid size and the virtual test area were designated individually for each brain region to adjust to the region of interest size and shape. Intraneuronal Aβ accumulation has been estimated by four neuropathologists in 12 brain structures including frontal, temporal and occipital cortex, amygdala, thalamus, lateral geniculate body, sectors CA1 and CA4, and dentate gyrus in the hippocampal complex, Purkinje cells and dentate nucleus in cerebellum, and inferior olive in the brainstem. The number of 4G8-negative neurons and neurons with weak (<10 immunopositive granules per cell), strong (condensed mass of indistinguishable small and large immunoreactive granules) and medium (>weak and <strong) immunoreactivity was determined using a ×40 objective lens. For each subject, from 100 to 180 neurons were examined per region of interest in sections immunostained with mAb 4G8. Inspection of the entire cell cytoplasm by using micrometer screw contributed to precise rating of amyloid load in each examined neuron.

Differences in the estimated cytoplasmic neuronal Aβ load were examined using the Mann-Whitney U (Wilcoxon signed ranks) test or, for comparison of all three groups, the Kruskal-Wallis one-way ANOVA (an extension of the U test) [90]. Statistics were computed from pooled data from each group [dup(15) autism, idiopathic autism, control], where sampled neurons immunoreactivity was categorized as strong, medium, weak or none.

Western Blotting

Frozen temporal cortex samples from three control and three autistic subjects were homogenized in 10×volume of 10 mM TRIS buffer containing 0.65% NP-40, 1 mM EDTA and

Table 2. Antibodies used for immunocytochemistry, immunofluorescence and western blotting.

Name	Epitope or target	Dilution	Host	Application	Source
6E10	4–13 aa Aβ	1:10,000	M-m	ICH,CM, WB	Covance, Inc., Princeton, Inc. [84,85]
6F3D	8–17 aa Aβ	1:50	M-m	ICH	Novocastra Laboratories Ltd., Newcastle, UK
4G8	17–24 aa Aβ	1:8,000	M-m	ICH,CM, WB	IBR [86]
Rabm38	−38 aa Aβ	100 ng/mL	R-m	ICH	IBR
Rabm40	−40 aa Aβ	100 ng/mL	R-m	ICH	IBR [87]
Rabm42	−42 aa Aβ	100 ng/mL	R-m	ICH	IBR [87]
R57	APP C-terminal aa 671–695	1:3,000	R-p	CM	IBR
R226	36–42 aa Aβ		R-p	CM, WB	IBR
R162	Aβ C-terminus		R-p	CM, WB	IBR
LAMP 1	Lysosomes	1:400	R-p	CM	Abgent, San Diego, CA
Rab5 Ab13253	Early endosomes	1:100	R-p	CM	Abcam Inc., Cambridge, MA
LC3B	Autophagic vacuoles	1:100	R-m	CM	Cell Signaling Technology Inc., Danvers, MA
COXIV	Mitochondria	1:100	R-m	CM	Cell Signaling Technology Inc., Danvers, MA
GFAP	Astrocytes	1:400	R-p	CM	Sigma, Saint Louis, MO
AIF/IBA1 AF1039C	Microglia	1:200	G-p	CM	Abgent, San Diego, CA
Tau-1	Tau protein	1:1000	M-m	ICH	IBR

Mouse monoclonal (M-m), Rabbit monoclonal (R-m) or polyclonal (R-p), Goat polyclonal (G-p). Immunocytochemistry (ICH), Confocal microscopy (CM), Western blots (WB).

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Complete protease inhibitor coctail (Roche, Mannheim, Germany) in a Potter-Elvehjem homogenizer and sonicated for 2 minutes. Protein content in lysates was measured by BCA assay (Pierce). Forty μg of total lysate proteins were loaded per lane for PAGE in 8–15% gradient gels.

Tissue samples from formalin-fixed PEG or celloidin-embedded brains of three subjects with diffuse plaques detected by immunocytochemistry (39-year-old female diagnosed with dup(15) autism, a 51-year-old autistic subject, and a 52-year-old subject with atypical autism) and two subjects without plaques (48-year-old autistic and a 47-year-old control subject) were used for protein extraction. From 50-μm-thick sections, approximately 120 mm^2 of affected cortex was dissected (approximately 6 mm^3 of tissue), rehydrated in PBS and homogenized in Potter-Elvehjem homogenizer in PBS containing 0.5% sodium deoxycholate, 0.1% SDS and 1% NP-40 (RIPA buffer). After sonication two times for three minutes, the material was centrifuged at 16,000g for 20 minutes, and supernatants were collected as RIPA extracts. Protein content in the extracts was measured by the BCA assay (Thermo Scientific, Rockford, IL). For Aß detection with R162, R226, and mAb 6E10, the amounts of extracted proteins loaded per lane were 3, 3 and 6 μg, respectively. The proteins were subjected to PAGE in 8–15% gradient gels, transferred onto nitrocellulose and probed with antibodies specific for C-terminus of Aß40 (R162) and Aß42 (R226), and N-terminus–specific mAb 6E10.

Supporting Information

Figure S1 Neurons with low and high amyloid load. In control brains, the percentage of Aβ-positive neurons and their amyloid load is much lower in CA1 than in CA4 sector and is very low in the granule neurons in the dentate gyrus. The percentage of Aβ-positive neurons and amyloid load is significantly higher in the dup(15) autism cohort than in the control and idiopathic autism groups ($p<0.0001$), but the difference between idiopathic autism and control is insignificant. The characteristic feature of the LGB, inferior olive and dentate nucleus of control subjects is the very high percentage of Aβ-positive neurons and the highest amyloid load among the examined 12 structures. The increase of amyloid load is undetectable in the inferior olive and is minimal in the LGB and dentate nucleus of subjects with idiopathic autism and dup(15) autism.
(TIF)

Figure S2 Topography and morphology of neocortical diffuse plaques. Low magnification demonstrates diffuse plaques immunostained with mAb4G8 (17–24 aa) in frontal, temporal and occipital cortex (FC, TC and OC, respectively) in the brain of a 39-year-old female diagnosed with dup(15) autism, a 51-year-old autistic male, and a 52-year-old subject with atypical autism.
(TIF)

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Author Contributions

Conceived and designed the experiments: Jerzy Wegiel BR MJL. Performed the experiments: JF BMK Jarek Wegiel AC VC. Analyzed the data: KN HI SYM MF. Contributed reagents/materials/analysis tools: DLM PDM. Wrote the paper: Jerzy Wegiel NCS EHC WTB. Genetic diagnosis: NCS EHC MS. Clinical evaluation: NCS EHC ILC EL. Neuropathological evaluation: TW IK Jerzy Wegiel.

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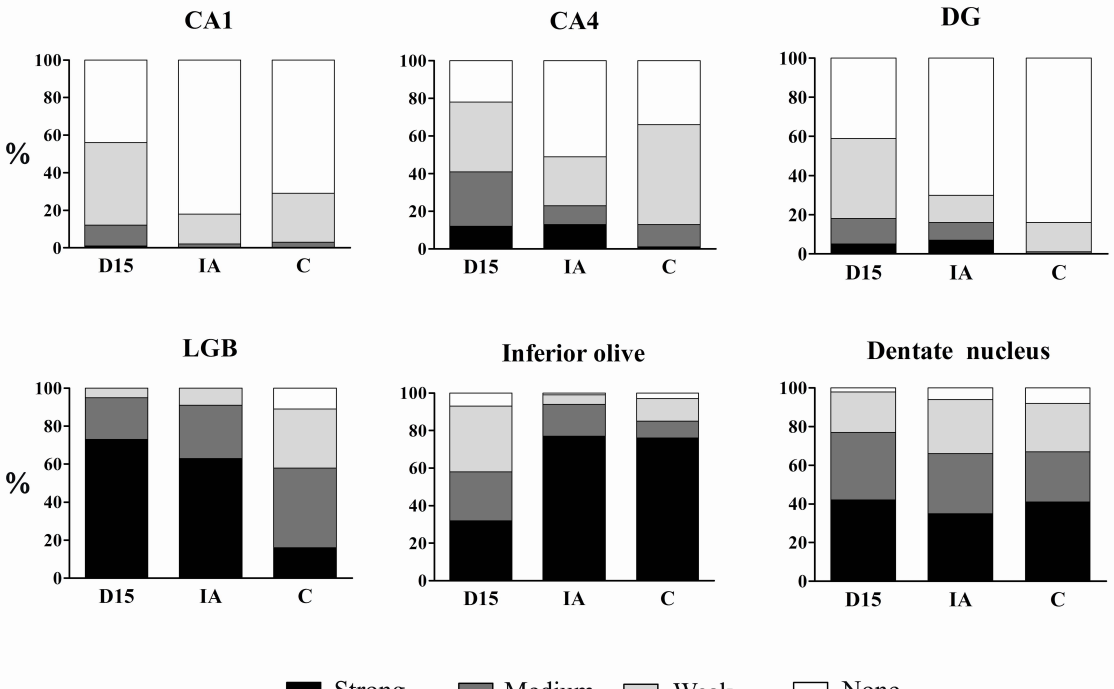
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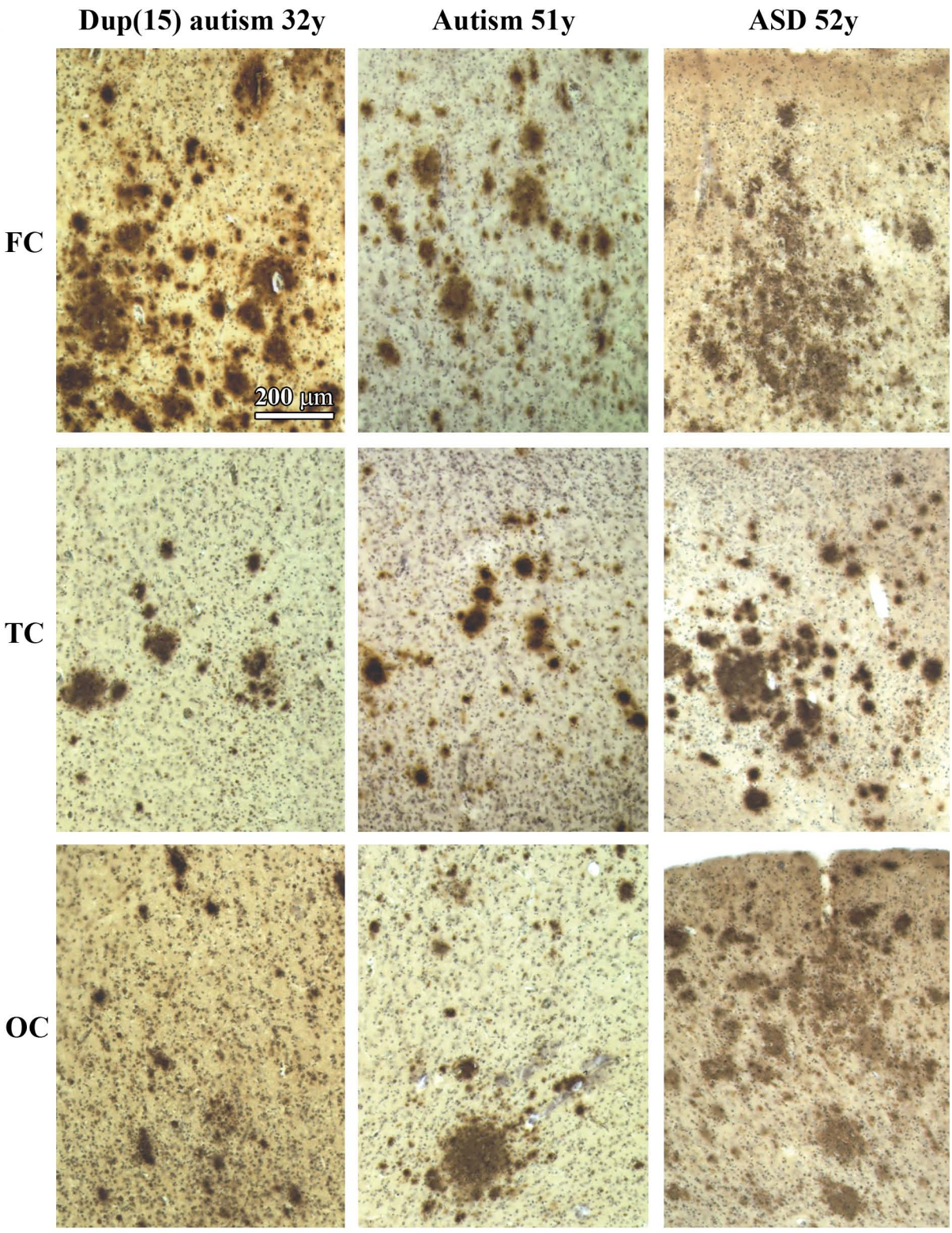
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Contribution of olivo-floccular circuitry developmental defects to atypical gaze in autism

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Summary

Individuals with autism demonstrate atypical gaze, deficits in facial perception, altered movement perception, and impairments in smooth persuit. Oculomotor functions are controlled by the olivo-floccular neuronal circuit. The aim of this study of the inferior olive in the brainstem and the cerebellar flocculus in 12 autistic and 10 control subjects was to determine the type and topography of developmental alterations that may contribute to a defective oculomotor function and atypical gaze in autism. The study revealed dysplastic changes in eight autistic (67%) and two (20%) control subjects. The dysplastic portion of the flocculus volume was small (5.5 mm^3 in autistic and 3.9 mm^3 in control subjects, on average) but alterations were extremely severe and included disorganization of the cytoarchitecture, deficit and altered morphology and spatial orientation of Purkinje, granule, basket, stellate and unipolar brush cells. These changes are indicators of profound disruption of flocculus circuitry and suggest severe functional alterations of the affected portion of the flocculus. In the flocculus of the autistic subjects, the volume of Purkinje cells was 30% less in the dysplastic than in the not-affected area (6,240 μm^3 and 8,230 μm^3, respectively; $p < 0.01$). Moreover, in the entire cerebellum of the autistic subjects the volume of Purkinje cells was 25% less than in the control subjects ($p<0.001$). These detected severe developmental abnormalities in the cerebellar flocculus, in the absence of changes in morphology and neuronal size in the inferior olive, suggest that floccular dysplasia plays a pivotal role in dysfunction of the oculomotor system in autism.

Key Words: Autism, Atypical gaze, Cerebellum, Olivo-floccular circuit, Dysplasia

INTRODUCTION

Autism is a severe and pervasive neurodevelopmental disorder characterized by deficits in social interactions, verbal and nonverbal communication, and restricted, repetitive behaviors (American Psychiatric Association, 1994). Among the most striking features of the social impairments in autism are deficits in coordinating visual attention with others.

Atypical gaze in autism. Eye gaze processing impairments appear early in the development of children with autism (Munday et al 1986; Dawson et al 1998). Even high functioning autistic subjects exhibit deficits performing tasks involving mental interferences from viewing expression in the eyes (Baron-Cohen et al 2001). Gaze processing deficits result from impairment in using gaze to understand the intentions and mental states of other people (Baron-Cohen 1995, Baron Cohen et al 1999a, 2001; Leekam et al 1998, 2000). Children with autism demonstrate "atypical" gaze and frequently have stereotypies including eye pressing, hand flicking and light gazing. Several studies have revealed impairments in smooth visual pursuit in autism (Rosenhall et al 1988; Scharre and Creedon, 1992; Takarae et al 2004a). A study of 34 autistic children showed that 31 children had atypical optokinetic nystagmus responses including delayed onset, short duration, gaze avoidance, or stereotypic behavior (Scharre and Creedon 1992). Autistic children had longer time constants during the primary nystagmus response and significantly fewer beats during the secondary response to vestibular stimulation, which may reflect brainstem dysfunction. Faulty modulation of sensory input and motor output within brainstem centers, may contribute to the disruption of more complex adaptive and motivated behaviors (Ornitz et al 1985). A study of 60 high-functioning individuals with autism revealed pursuit eye movement deficits (Takarae et al 2004a). The subjects had a reduced closed-loop pursuit gain when tracking both oscillating and ramp targets. Deficits were more apparent after mid-adolescence suggesting there is a reduced maturational development of the pursuit system in autism. Bilateral disturbances in the ability to use internally generated extraretinal signals for closed-loop pursuit suggests there are defects in the frontostriatal or cerebellar circuitry. An fMRI investigation using saccadic and pursuit eye movement paradigms revealed in high functioning autistic subjects that there was reduced activation in cortical eye fields and cerebellar hemispheres (Takarae et al 2007). Increased activation in the prefrontal-striatal-thalamocortical circuitry during visually guided saccades suggests that systems dedicated

to cognitive control may need to compensate disturbances in the lower-level sensorimotor systems.

The role of flocculus in eye movement control. Experimental studies indicate that the cerebellar flocculus is involved both in the vestibulooccular reflex arc and olivocerebellar circuit. The flocculus exerts a specific inhibitory modulation of both excitatory and inhibitory branches of the vestibuloocular reflex pathways to the extraocular muscles. This modulation is mediated through inhibitory projections of floccular Purkinje cells to the vestibular nuclei neurons, described as flocculus target neurons (reviewed by du Lac et al 1995; Sato and Kawasaki 1991). The flocculus is also a region of vestibular afferent signal convergence through the input of mossy fibers from the vestibular nuclei and of visual signals received via the climbing fibers of the inferior olive (Sato and Kawasaki 1991).

A substantial number of Purkinje cells in the flocculus of the monkey receives converging visual inputs from functionally distinct portions of the retina and subserve the neural mechanisms for oculomotor controls during slow eye movements. The flocculus provides the occulomotor system with eye position information during fixation and with velocity information during smooth pursuit and participates in the control of oculomotor functions (Noda and Suzuki 1987, Zee et al 1981). Velocity plays a stronger role (64%) than position (36%) in determining firing rate modulation during circular pursuit (Leung et al 2000). The majority of visual Purkinje cells (85%) are identified as horizontal gaze-velocity neurons. The presence of position and velocity signals in the flocculus and paraflocculus suggest that these two cerebellar regions generate the position and velocity signal that are used to control eye motion (Leung et al 2000). Moreover, Purkinje cells in the floccular region help to coordinate eye and head movements during active gaze shifts by modulating the vestibulo-ocular reflex (Belton and McCrea 1999).

Olivo-cerebellar system. From a movement perspective, the olivo-cerebellar system has been considered to be involved in motor learning (Ito 2001, Hesslow and Yeo, 2002, Highstein et al 2005) and in the timing of motor execution (Lang et al 1999). The olivo-cerebellar system is involved in both somatomotor and oculomotor cerebellar control. Electrophysiological studies have revealed that neurons in the dorsal cap of Kooy (DCK) and the adjacent ventrolateral outgrowth (VLO) of the inferior olive control eye movements via their climbing fiber projection to the cerebellar flocculus; whereas neurons in the principle olive are involved in control of limb and digit movements via their climbing fiber projections to the lateral part of the cerebellar

hemisphere (reviewed by Voogd and Bigare, 1980; and Ito, 1984). Oculomotor inferior olive neurons have more distinctive electrophysiological phenotypes compared to somatomotor neurons of the inferior olive. Differences in the motor control requirements between somatic and ocular movement have led to fundamental differences in the electrophysiological properties of DCK/VLO neurons and principle olive neurons and a unique specialization of the DCK/VLO – flocculus circuit (Urbano et al 2006).

Neuropathological studies indicate that the oculomotor circuit is often developmentally affected in autistic subjects. Our study of serial sections of the brain, including the cerebellum, revealed multiregional dysregulation of brain development in 12 of 13 (92%) autistic subjects. The presence of cerebellar floculonodular dysplasia in six subjects (46%), focal dysplasia in the vermis of one subject, heterotopia in one case and focal hypoplasia in one subject, reflected a high susceptibility of the cerebellum to developmental defects in autistic subjects (61% affected by one or more changes), and especially of the flocculus (Wegiel et al 2010). The presence of olivary dysplasia in three of five autistic subjects and ectopic neurons related to the olivary complex in two cases reported by Bailey et al (1998) suggests that both components of the oculomotor circuitry are prone to developmental defects.

Aim. The aim of this study was to detect defects of the olivo-cerebellar circuitry that may be responsible for altered oculomotor activity, atypical gaze, deficits in facial perception, altered movement perception, and impairments in smooth pursuit observed in autism.

MATERIAL AND METHODS

The cerebellum and brainstem of 12 autistic and 10 control subjects were examined (Table 1). Tissue acquisition was based on individual tissue transfer agreements with several tissue banks including: (a) the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, (b) the Harvard Brain Tissue Resource Center and (c) the Brain and Tissue Bank for Developmental Disabilities and Aging of the New York State Institute for Basic Research in Developmental Disabilities. The Institutional Review Board of the New York State Institute for Basic Research in Developmental Disabilities approved the study. Tissue samples and case clinical records were identified with a codified brain bank number.

One brain hemisphere from each subject was fixed in 10% buffered formalin for a period of time ranging from 6 weeks to several months, dissected into 30 mm thick frontal slabs, dehydrated in a

graded series of ethyl alcohol and embedded in celloidin as described (Wegiel et al 2010) or in polyethylene glycol (PEG) as described (Wegiel et al 2011). The cerebellum and brainstem were cut in a saggital plane. 200 μm-thick celloidin and 50-μm thick PEG serial hemispheric sections were used for morphometry. Free-floating 50 μm-thick serial PEG sections were used for immunostaining.

Inclusion and exclusion criteria. The application of the Autism Diagnostic Interview-Revised (ADI-R) eliminated cases of atypical autism (Wegiel et al 2010, 2011). An expanded neuropathological protocol, based on examination of one cresyl violet (CV) stained section per 0.6 mm in the celloidin protocol and per 1 mm in the PEG protocol, was used to detect the type, topography, and severity of qualitative changes. The application of neuropathological criteria eliminated cases with pathology not related to autism, including pre- and postmortem changes. The results of the neuropathological, immunocytochemical and morphometric studies of the inferior olive and cerebellum of autistic and control subjects are reported here.

Quantitative methods. To detect quantitative changes, an image analyzer with a computer-controlled automatic stage installed on an Axiophot microscope (Zeiss) and a stereology software package (Microbrightfield, VT, USA) were used. The volume of the flocculus and subdivisions (molecular and granule layer and white matter) were estimated at 48x magnification (objective lens x1.25) using Stereo-Investigator (Microbrightfield). The border between subdivisions was verified at 184x magnification (objective lens x5). The volume of the Purkinje cells soma and nuclei were estimated in the flocculus and in the entire cerebellar cortex at 1,480x magnification (objective lens x40) using Nucleator (Microbrightfield). On average, 13 sections with 1-mm intervals in autistic subjects and 14 in control subjects with 1-mm intervals were used for the morphometric examination.

Immunostaining. Several antibodies were employed to detect and characterize neuronal and glial developmental alterations in the flocculus as indicated in Table 2. Anti-calbindin mouse mAb D-28k was used to characterize the topography and morphology of the Purkinje cell soma, dendritic tree and axons (Table 1). Rabbit polyclonal anti-parvalbumin antibody was employed to characterize the morphology of the Purkinje cells, basket cells in the internal one third of the molecular layer, and the stellate cells in the external two third of the molecular layer of the cerebellar cortex. Unipolar brush cells (UBCc) in the granule cell layer were detected with an anti-calretinin mouse mAb Ab-1. Astrocytes were immunostained with a goat anti-GFAP polyclonal antibody. Projections of the raphe nuclei serotonergic neurons ere detected in the flocculus with mouse mAb ST51-2. Anti-mouse HRP/DAB

detection kit (ab64259, Abcam Inc., Cambridge, MA) was used to enhance immunoreactivity of the serotonin transporter positive neuronal processes.

RESULTS

Prevalence of dysplasia in cerebellar nodulus and flocculus. Dysplasia of the cerebellar flocculus was detected 3.3 times more often in the autistic subjects (8/12; 67%) than in the controls (2/10; 20%). Dysplasia was also found in the nodulus but abnormalities were equally common in autistics (9/12; 75%) and controls (8/10; 80%), and the affected area was smaller than in the flocculus.

Quantitative characteristics of the flocculus and Purkinje cells. The average volume of the flocculus was greater in the autistic (224 mm^3) than in the control subjects (179 mm^3; $p<0.007$). The average volume of dysplastic area in autistic subjects was greater (5.5 mm^3) than in controls (3.9 mm^3), but the difference failed to reach significance. Significant inter-individual differences in the volume of the dysplastic area were observed among eight autistic subjects (from 0.2 to 14.7 mm^3) and in two control subjects (0.5 and 7.3 mm^3). The dysplastic area had modified proportions between the major constituents. There was much less of a contribution of the molecular layer (29%) in the dysplastic than in the unaffected area (40%), with approximately equal contributions of the white matter (13% in dysplastic and 14% in unaffected area), and a larger volumetric contribution of the granule cell layer (58% in dysplastic and 46% in the unaffected area).

Application of the Nucleator revealed that the volume of the Purkinje cells in eight autistic subjects was reduced in the dysplastic area by 30% (6,240 μm^3) in comparison to the non-dysplastic area (8,230 μm^3)($p< 0.01$). In two control subjects with dysplastic changes, the difference between the volume of Purkinje cells in the dysplastic (7,477μm^3) and the non-dysplastic (9,367 μm^3) areas was 20% ($p < 0.01$). The volume of Purkinje cells in the entire cerebellum was 25% smaller in 7 autistic subjects (8,382 μm^3) than in 7 control subjects (11,268 μm^3)($p<0.001$) (celloidin protocol only).

In contrast to a significant reduction of Purkinje cell volume in the cerebellum of the autistic subjects, CV-based morphometry of the inferior olive neurons revealed a similar cell soma volume in the autistic (4,018 μm^3) and control (4,012 μm^3) subjects.

Topography and morphology of floccular dysplasia in autism. Dysplastic changes were observed in the most rostral portion of flocculus (Fig. 1 a-e). There was profound distortion

of the laminar organization of the granule, molecular and Purkinje cell layers in the dysplastic portion of the flocculus. Granule cells did not form a layer but rather islands and a labyrinth of a thin irregular-shaped cellular septa. The granule layer was mixed with an altered molecular layer and small islands of white matter. Purkinje cells were few and dispersed in an abnormal granule and molecular layer, and in the white matter. As in the flocculus, dysplastic changes in the nodulus were also located in the rostral portion (Fig. 1f, g).

Defects of flocculus cytoarchitecture. In the unaffected portion of the flocculus, immunocytochemistry using anti-calbindin mouse mAb D-28, revealed that the Purkinje cells dendritic tree was the major factor determining the volume and structure of the molecular layer where (Fig. 2a) their axons penetrate the granule cell layer. Parallel bundles of Purkinje cells axons are an integral component of the cytoarchitecture of granule cell layer and white matter (Fig. 2 b, c).

As striking as the deficits in Purkinje cells (Fig. 2d-e), defects in the development of their dendritic trees and abnormal spatial orientations appear to be some of the major factors determining the structural defects of the dysplastic flocculus. Four major types of Purkinje cells developmental defects were observed (Fig. 2g-i). (1) Some Purkinje cells, dispersed among loosely arranged granule neurons, were monopolar with a distinct axon and usually with thicker and thinner segments. These Purkinje cells did not develop dendritic trees and this defect appeared to be a major contributor to the deficit and abnormal morphology of the molecular layer in the dysplastic flocculus. (2) A few Purkinje cells formed dendritic trees but branches were significantly truncated and spatially distorted. (3) Other Purkinje cells lost polarity and formed long irregular processes radiating in all directions. (4) The fourth type of abnormal Purkinje cells had very numerous but short (30-40 μm) dendrites forming a dense corona around the cell body.

Immunostaining with anti-calbindin (Fig. 2), and especially with anti-parvalbumin antibodies (Fig. 3), revealed not only severe dendritic abnormalities but also severe alterations of the Purkinje cell axons in the dysplastic areas. Almost all axons showed alternative thickening and thinning giving the axons a bead-like morphology. Moreover, immunostaining with anti-parvalbumin antibody revealed almost a complete lack of basket and stellate neurons in the molecular layer in the dysplastic flocculus (Fig. 3). The study of distribution of calretininn-positive UBCs revealed enhanced accumulation of UBCs in the flocculus of control subjects in comparison to other cerebellar folia (Fig. 4). However in the

dysplastic area of the flocculus of autistic subjects severe deficits of calretinin-positve UBCs and 2-3 fold increased diameter of numerous axons of these cells were observed. Severe alterations of the number, distribution and morphology of all types of neurons in the dysplastic portion of the flocculus was associated with an increase in the number of Bergmann glial cells, enhanced immunoreactivity of the cell cytoplasm, and spatial disorientation of the Bergmann fibers (Fig. 5).

In the portions of the nodulus and flocculus unaffected by developmental alterations, a few serotonin transporter immunopositive (ST51) axons transversed the granule cells and molecular layer. Two or three branches of the ST51-positive fibers surrounded the bodies of individual Purkinje cells. In contrast to moderate serotonergic innervation in the unaffected portion, the dysplastic area was characterized by a several-fold increase in the number of ST-51-positive fibers. The largest serotonergic bundles of fibers were observed on the periphery of the dysplastic areas, frequently mixed with loosely arranged cells with the morphology of granule cells. These islands of granule-like cells frequently merged with a ring of granule cells around the dysplastic area. A characteristic feature of the loose and compact serotonergic fibers was a large number of varicosities in fibers in the abnormal granule cells and molecular layer (Fig. 6).

DISCUSSION

Cerebellar pathology in autism. Neuroimaging and neuropathological studies indicate cerebellar abnormalities are one of the most consistent finding in autism (Ritvo et al 1986, Bauman and Kemper 1996, Kemper and Bauman 1993, Whitney et al 2008, 2009, Courchesne et al 2001). Several studies have indicated abnormalities of cerebellar vermis function in autism (Takarae et al 2004b, Pelphrey et al 2005). MRI studies have shown smaller vermian lobules VI-VII, which are likely due to neuronal hypoplasia (Hashimoto et al 1995, Bauman and Kemper 1996, Courchesne et al 1989, 2001, Kaufmann et al 2003). A review by Palmen et al (2004) noted a reduced number of Purkinje cells to be present in 72% of the reported autism cases. However, the immunostaining for calbindin has indicated that the percentage of autistic subjects with a reduced number of Purkinje cells is in the range of 33% to 50% (Whitney et al 2008). In contrast to the hypoplasia hypothesis Whitney et al (2008, 2009) postulated that the reduced number of PCs is not the effect of a developmental deficit. The authors reported that PCs were generated, migrated to their proper location site and subsequently died. In autism, we have found the cerebellum is frequently affected by several types of developmental abnormalities including defects of migration with heterotopias, focal nodulus and flocculus dysplasia,

other forms of vermis and cerebellar folia dysplasia, and sporadically by focal polymicrogyria (Wegiel et al 2010, 2011). This broad spectrum of structural changes appears to define a high vulnerability of the cerebellum to developmental alterations and this may contribute to the autism phenotype. Reported here, the dysplastic changes in the flocculus appear to be a peculiar form of severe focal developmental defect resulting in profound alterations of the cytoarchitecture and a disorganization of the neuronal circuits in this cerebellum subregion, that possess a unique and narrow function. The study suggests that the unique pattern of developmental alterations, different than other forms of cerebellar cortical dysplasia, reflects a unique etiology and mechanism, which may cause unique functional alterations.

Prevalence of floccular dysplasia in autism. In the examined cohort of subjects diagnosed with idiopathic autism, flocculus dysplasia was found in 8/12 subjects (67%). A similar prevalence of floculus dysplasia (6/8; 75%) was also detected in a cohort with autism caused by chromosome 15 duplication (Wegiel et al 2011). This suggests that these alterations develop in autistic subjects, regardless of etiology (Wegiel et al 2011). A very small and moderate size of floccular dysplasia was also found in 2/10 control subjects. Characteristics of possible clinical correlates for these structural flocculus defects, including measures of defective oculomotor function, atypical gaze, eye movement pursuit deficits in autistic, as well in control subjects, are not available. The absence of clinical signs in the control subjects with some dysplastic changes may indicate that defects of flocculus cytoarchitecture and connectivity are clinically expressed only when are large enough and combined with other defects. It may suggest that, for clinical expression, at least five major potential correlates should be considered including: the presence, size and topography of (1) the dysplastic changes in the flocculus and (2) nodulus; developmental alterations (3) in the inferior olive and (4) in the vestibular nuclei; and the presence, topography, and severity of developmental alterations of (5) Purkinje cells in the entire cerebellum.

The role of Bergmann glia in spatial disorganization in the dysplastic flocculus. The profound spatial disorganization of the granule, molecular, and Purkinje cell layer indicates that the mechanisms controlling migration during flocculus development are significantly altered in the rostral flocculus portion of majority of autistic subjects. Neuronal migration in different domains of the cerebellar cortex is regulated by domain-specific factors. Granule cell migration from the external granular layer to the internal granule layer is guided by surface-mediated interactions with Bergmann glial fibers, which traverse the developing molecular layer, and

migrate on the fascicles of the ascending axons of earlier generated granule cells (Rakic 1971, 1981). However, migration to a final destination within the granule layer is modulated in response to local environmental cues (Komuro and Rakic 1998). The lack of parallel Bergmann fibers and the spatial disorientation of their processes in the molecular layer of the dysplastic flocculus indicate that they were not able to serve as the guide for granule cells. The result is a profound defect of granule cell arrangement.

Experimental studies indicate that developmental deficits of the Bergmann glia, alterations of their distribution, and abnormal length of their processes affect cerebellar architecture and that the severity of Bergmann glia defects correlates with misplacement of cortical cells and alterations of cortical layering (Kaartinen et al 2001, Qu and Smith 2005, Yue et al 2005). In neonataly X-radiated rats (Ferguson 1996, Li et al 2006) and rats treated with methylazoxymethanol (MAM) (Lafarga et al 1998) or cisplatin (Pisu et al 2005) alterations of the Bergmann radial fibers result in abnormal migration and ectopic displacement of granule cells in the molecular layer. In GFAP-Sox transgenic mice, failure of radial glia to extend radial fibers to the pial surface, and their abnormal migration results in abnormal migration of cortical neurons and aggregation in clusters (Hoser et al 2007).

Moreover, Cerri et al (2010) postulated that abnormal migration of granule cells result in alteration of the guiding role of granule cells fibers terminating on Purkinje cells and alters Purkinje cell dendritic tree development. PD 10 is considered a critical phase in Purkinje cell differentiation (Pisu et al 2003). Changes in the postnatal growth and remodeling of Purkinje cell dendrites in the postnatal period has been reported after X-ray irradiation or treatment with cytotoxic substances (Ferguson 1996, Avella et al 2006, Li et al 2006) and in mutant mice (See Katsuyama and Terashima 2009).

Flocculus pathology observed in autism might also be associated with foliation defects. Foliation patterning is genetically determined (Altman and Bayer 1997). The crucial event of the initial formation of cerebellar fissures is the formation of anchoring centers at the base of developing cortical fissures (Sudarov and Joyner 2007). In the anchoring centers, a basic role is assumed by the granule cells and Bergmann glial fibers to direct migration of granule cells at the base of the fissure. Purkinje cells secrete Shh which regulates the number of folia via regulation of granule cell proliferation in the external granule layer (Corrales et al 2004, 2006). Engrailed2 (En2) regulates the morphogenetic clock, that normally controls the timing of genetic and

cellular events that direct formation of the anchoring centers (Sudarov and Joyner 2007). Rat studies reveal evidence that development of cortical anomalies occurs during postnatal life. The first signs of pathology - retraction of Bergmann radial glia, distortion of their processes and lack of endfeet - are detected in 40% of rats at 10 days of life, in the stage of active granule cell proliferation and migration (Necchi et al 2000)

A striking deficit of Purkinje cells, the reduction of granule cells, a severe deficit of unipolar brush cells in the granule cell layer and the granule cell islands, an almost complete lack of inhibitory basket and stellate cells in the molecular layer are indicators of structural defects of neuronal circuits in the flocculus of a majority of the idiopathic autistic subjects and those with dup(15) autism.

UBCs abnormalities in dysplastic flocculus. Flocculus belongs to cerebellar structures with the highest numerical density of UBCs observed in examined human samples and in different species of mammals (Dino et al 1999). UBC-rich region extends to nodulus and in animals to paraflocculus. This topography is consistent with data indicating that flocculus extends beyond its anatomical borders to a "flocculus-like" region of the ventral paraflocculus (Tan et al 1995). Experimental studies indicate that flocculus and nodulus play antagonistic roles in learning and timing of eye movements (Koekkoek et al 1997). These data support Dino et al hypothesis (1999) that UBCs are involved in the control of movements and postural adjustments dependent on vestibular, proprioceptive and ocular stimuli, including vestibulooccular reflexes (VOR) controlling eye movements.

This study indicates that a small portion of the nodulus, most likely anatomically related to the flocculus is affected by dysplastic changes in the majority of control and autistic subjects, whereas flocculus is affected in 67% of autistic and small changes are observed in only 20% of control subjects. Dysplastic flocculus reveals severe deficits of calretinin-positive UBCs, and severe axonal pathology in these neurons. One may hypothesize that these developmental defects contribute to a disruption of both, the structure and the function of the UBC/granule cell/Purkinje cell circuits. The UBCs are excitatory interneurons located in the granule cell layer (Dino et al 2000a, Ito 2006, Mugnaini and Floris 1994, Nunzi et al 2001). The majority of UBCs have a single dendritic process terminating with a compact arrangement of short dendrioles forming a brush-like structure. These cells receive glutamatergic input on the brush from an individual mossy fiber (Dino et al 2000a). Mossy fibers synapses facilitate fast and peculiarly slow currents

mediated by alfa-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors (Mugnaini et al 1994, Rossi et al 1995). The UBCs identified by calretinin immunoreactivity express the glutamate receptor subunits GluR2/3, NMDAR1, and mGluR2/3. They provide a powerful network of feed-forward excitation within the cerebellar granular layer, which may amplify vestibular signals and synchronize activity in clusters of functionally related granule cells, and patches of Purkinje cells innervated by vertical projections of granule cells (Nunzi et al 2001). UBCs receive input from the vestibular nuclei and the vestibular ganglion (Dino et al 2000b). They project their axons within the granular layer (Berthie and Axelrad 1994, Rossi et al 1995, Dino et al 2000a). Collateral branches of the UBC axon terminate in glomerular rosettes (Dino et al, 2000a). The primary function of UBCs may be to provide a synchronized excitatory drive to clusters of granule cells, which in turn drive the activity of patches of PCs whose dendrites lay in the ascending path of the granule cell axon (Nunzi et al 2001). Patches of synchronously active PCs reflect the activity of underlying granule cell clusters that exert a strong excitatory drive by means of the axonal branch, which terminates on the most distal portions of the PC dendritic tree (Llinas 1982, Cohen and Yarom 1998, Gundappa-Sulur et al 1999).

Originally, UBCs were thought to be products of the ventricular zone of the fourth ventricle but Englund et al (2006) found that their origin is in the rhombic lip. During cerebellar development, *Disabled homolog 1 (Dab1)* mediates Purkinje cell dispersal into a monolayer. *Dab1* disruption results in the failure of dispersal and a loss of foliation (Howell et al 1997, Gallagher et al 1998). Experimental data suggest that UBCs become regionally restricted through interactions with the embryonic array of Purkinje cell subtypes (Chung et al 2009). Therefore UBCs early developmental defects may initiate or contribute to the cascade of alterations resulting in flocculus developmental abnormalities and contribution to clinical signs of autism.

Deficit of inhibitory interneurons: basket and stellate cells. Purkinje cells function is dependent upon their innervation by the GABAergic basket cells and stellate cells in the molecular layer. Both, basket and stellate cells are parvalbumin-positive GABAergic interneurons residing in the molecular layer of the cerebellar cortex and innervating Purkinje cells. Basket cells are located in the lower one-third of the molecular layer and their axons contribute to the plexus surrounding Purkinje cells soma (Sultan and Bower, 1998). Stellate cells, located in the upper two-thirds of the molecular layer, innervate Purkinje cell dendrites (Palay

and Chan-Palay 1974). These two types of interneurons receive excitatory input from granule cell parallel fibers and inhibitory input from the Lugaro cells residing in the granule cell layer (Laine and Axelrad 1998) and other basket and stellate cells (Palay and Chan-Palay 1974). Inhibitory innervation by basket and stellate cells plays an important role in Purkinje cell function, however the reduction of Purkinje cell numbers observed in the cerebellum of autistic subjects (Whitney et al 2008), does not affect the density of basket or stellate cells detected by immunohistochemistry for parvalbumin (Whitney et al 2009). The deficit of basket and stellate neurons observed in the dysplastic flocculus suggests focal defects of the inhibitory system development in the olivofloccular circuitry in the majority of autistic subjects.

Abnormal serotonergic innervation of dysplastic flocculus and nodulus. Studies of the human cerebral cortex have indicated that 5-HT innervation patterns are normal in cryptogenic epilepsy. However, serotonergic hyper-innervation in focal cortical dysplasia indicates that developmental brain epileptogenic lesions are associated both with abnormal cytoarchitecture and altered neuronal circuitry, including focal alterations of the serotonergic fibers (Trottier et al 1996). One may assume that focal serotonergic hyper-innervation in the dysplastic flocculus is a response to defective development of a portion of the flocculus and expresses the sprouting of serotonergic fibers to build connections with missing Purkinje cells, basket and stellate cells, as well as granule cells. However, the presence of severe cerebellar cortical dysplasia in vermal lobe IX in one of examined subjects with the absence of Purkinje cells, interneurons (basket and stellate cells) and an almost total lack of serotonergic fibers in this dysplastic area suggests that developmental deficit of targets for serotonergic innervation is not a trigger for the compensatory sprouting and serotonergic hyperinnervation in lobe IX. The absence of serotonergic hyper-innervation in focal polymicrogyria, another form of cortical dysplasia in the same cerebellum, also suggests that compensatory sprouting is also not triggered by mechanisms leading to cerebellar polymicrogyria.

The cerebellar cortex, receives serotonergic innervation in the form of a plexus of fine varicose fibers that do not face any postsynaptic element. Serotonin is released through volume transmission and is acting as paracrine agent (Bishop et al 1993, Trouillas and Fuxe 1993). The striking increase in the number of varicose fibers in the dysplastic flocculus appears to be a sign of enhanced release of serotonin in this cerebellar structure already severely affected by developmental abnormalities. The serotonin level in the cerebellar cortex is specifically and

positively correlated to the level of motor activity (Mendlin et al 1996). Because serotonin selectively excites Lugaro cells, one may expect a modification of Golgi cell inhibitory input on large populations of granule cells, and indirectly on Purkinje cells (Dieudonne and Dumoulin 2000). The oculomotor activity abnormalities observed in autism could be the collective product of severe changes of flocculus circuits and exposure of this dysfunctional system to excessive amounts of serotonin.

A comparison of the morphology of three types of developmental defects: (a) flocculus and nodulus dysplasia, (2) focal severe underdevelopment of cerebellar cortex in lobe IX, and (c) focal polymicrogyria in the other lobe coexisting in a single brain, indicates that serotonergic hyper-innervation is strikingly strong in flocculus/nodulus dysplasia with a unique pattern of cytoarchitectural disorganization, neuronal deficits, and a disruption of the basic organization of cerebellar circuitry. The presence of strong serotonin transporter immunoreactivity (a) within bundles of fibers passing by the flocculus and nodulus in the cerebellar pedunculi and providing serotonergic innervation to all cerebellar subdivisions, (b) in bundles of these axons encompassing the nodulus and flocculus and (c) in bundles of these axons entering in the dysplastic area, may indicate that serotonergic hyper-innervation is associated with unique mechanisms of flocculus and nodulus dysplasia resulting in a unique morphology, cytoarchitectural disorganization, and circuitry defects with an abundance of serotonin fibers in these cerebellar region.

Spatial disorganization of the cortex within a dysplastic flocculus is the reflection of a very severe disruption of neuronal circuits critical for normal occulomotor activity and gaze control. This may contribute to the mental and social effects due to the lack of or limited eye contact of autistic subjects with other people.

Figures

Fig. 1. Dysplastic changes in the rostral portion of the flocculus of a 36 year old autistic subject (AN16961). Low magnification (a) illustrates the proportions between the size of cerebellum, flocculus (large arrowhead), and dysplastic area (small arrowhead). Four serial sections show loss of spatial organization of the granule and molecular layers in the dysplastic portion (small arrowheads) of the flocculus of this subject (b-e). Fig f and g illustrate the topography and morphology of dysplastic changes (small arrowhead) in the rostral portion of the nodulus of a 7 year old autistic subject (AN13961) (200 μm-thick celloidin sections).

Fig. 2. Anti-calbindin immunostaining with mouse mAb D-28k in the non-affected portion of flocculus reveals dendritic tree of Purkinje cells (a), morphology of axon (arrowhead) penetrating granule layer (b), and bundles Purkinje cells axons in the white matter (c). In the dysplastic flocculus, irregular islands of granule (G) neurons are mixed with a poorly defined molecular (M) layer, a few small calbindin-positive Purkinje cells and bundles of their axons (d and e). Almost all Purkinje cells display abnormal morphology of axons with an alternative increase and reduction of the axon diameter (bead-like axons) (f), several types of dendritic tree abnormalities including truncated dendritic tree (g), bipolar Purkinje cells (h; small arrowhead) and Purkinje cells with numerous short non-branching dendrites (h, large arrowhead); and Purkinje cell with spatially disoriented non-branching dendrites (i).

Fig. 3. Anti-parvalbumin immunostaining in the non-affected portion of the flocculus revealed soma and dendritic tree of Purkinje cells, basket cells (small arrowhead) in the internal one third of the molecular layer and stellate cells (large arrowhead), in the external two-thirds of the molecular layer (a). In the dysplastic areas, a few small Purkinje cells (large arrowheads), almost without dendritic tree, and a lack of both types of interneurons (basket and stellate cells) are observed (b). In non-affected flocculus, axons of Purkinje neurons (pairs of small arrowheads) in the granule cells layer (c) and in the subcortical white matter (e) are numerous and have normal morphology. Axons (pairs of arrowheads) in the dysplastic flocculus show significant swelling and thinning in the granule layer (d) and subcortical white matter (f)

Fig. 4. Anti-calretinin immunostaining revealed typical number and distribution of the unipolar brush cells in the granule cell layer in lobe 2 (a). CA-positive UBC have a single dendritic process terminating with a compact arrangement of short dendrioles forming a brush-like structure (b). In the non-affected flocculus, the number of UBCs is several times more than in other cortical regions (c, d). In the dysplastic flocculus, the number of UBCs is much less (e), their axons are much thicker (f) and locally clustered (g), whereas in other areas of the dysplastic granule layer UBCs and their axons are missing (h).

Fig. 5. Anti-GFAP immunostaining shows numerous straight Bergmann fibers (arrowheads) in the Purkinje cell-rich cortex of the control subject (a, b). In autistic subject cerebellum, in areas with reduced number of Purkinje, Bergmann cells are more numerous (arrowheads; c) and larger (d) than in control subjects. Their processes are abnormally numerous and twisted (c). In the dysplastic portion of the flocculus of the autistic subject, the Bergmann cells are dispersed (arrowheads; e) or clustered (f) in the molecular layer. Their soma is strongly GFAP-positive (arrowheads; f). Their processes do not have any spatial orientation (e and f).

Fig. 6. Immunostaining with ST51 revealed serotonin transporter in fibers in the molecular and granule cells layers (a) in non-affected portion of the flocculus. Immunostaining is abnormally strong in bundles of fibers (arrowheads) located between the ependymal cell layer and dysplastic portion of the nodulus (b) and flocculus (c). Higher magnification shows granule cells clusters perforated by dense (d) bundles of ST51-positive fibers. Purkinje (e) and granule cells (f) in dysplastic flocculus are surrounded by ST51-positive fibers with numerous varicosities.

Table 1. Material and the volume of the flocculus dysplastic and non-dysplastic area.

No	Group	Brain Bank number	Age (yrs.)	Sex	Hem	Embedding	Flocculus volume (mm^3)			Dysplasia: % of total volume
							Total	Non-affected	Dysplastic area	
1	A	IBR-425-02	4	M	R	Celloidin	221	220	0.2	0.1
2	A	AN13961	7	M	R	Celloidin	225	213	11.5	5.1
3	A	HSB-4640	8	M	R	PEG	217	214	3.6	1.7
4	A	AN01293	9	M	R	PEG	174	174	0.0	0.0
5	A	B-5223	15	M	R	PEG	287	285	2.3	0.8
6	A	AN09730	22	M	R	Celloidin	240	236	4.2	1.7
7	A	AN08166	28	M	R	PEG	303	303	0.0	0.0
8	A	NP06-54	32	M	L	PEG	179	179	0.0	0.0
9	A	AN16961	36	M	R	Celloidin	228	210	17.3	7.6
10	A	AN08105	56	M	R	Celloidin	270	258	12.1	4.5
11	A	AN09714	60	M	R	Celloidin	170	170	0.0	0.0
12	A	AN12698	66	M	R	Celloidin	198	192	6.5	3.3
						Average	226	221	7.2	2.1
1	C	AN2456	4	F	R	Celloidin	177	177	0.0	0.0
2	C	UMB-4898	7	M	R	Celloidin	148	148	0.0	0.0
3	C	UMB-1708	8	F	R	Celloidin	224	224	0.0	0.0
4	C	UMB-1670	13	M	R	PEG	176	176	0.0	0.0
5	C	UMB-4722	14	M	R	PEG	158	144	14.1	8.9
6	C	UMB-1846	20	F	R	Celloidin	154	154	0.0.	0.0.
7	C	UMB-4543	29	M	R	Celloidin	170	170	0.0.	0.0.
8	C	UMB-1576	32	M	R	Celloidin	196	195	1.6	0.8
9	C	IBR-291-00	32	M	R	PEG	187	187	0.0	0.0
10	C	AN11184	64	M	R	Celloidin	203	203	0.0	0.0
						Average	179	178	7.8	1.0

The average dysplastic area volume and percentage was calculated only for cases with dysplasia.

Table 2. Antibodies used for characterization of cerebellar flocculus dysplasia in autism.

Antibody	Target protein/cells	Host	Dilution	Pretreatment	Producer/vendor
Anti-Calbindin D-28k	Purkinje cells	Mouse mnoclonal	1:1000	No	Swant; Bellinzona, Switzerland
Parvalbumin	Purkinje, basket and stellate cells	Rabbit polyclonal	1:1000	No	Swant; Bellinzona, Switzerland
Calretinin Ab-1 (Clone 5A5)	Unipolar brush cells	Mouse monoclonal	1:100	10 min microwaving in citric acid	Thermo Fisher Scientific, Fremont, CA
GFAP	Mature Bergmann glial cells	Goat polyclonal	1:500	No	Santa Cruz Biotechnology; Santa Cruz, CA
ST51-2 Ab45525	Serotonergic projections from raphe nuclei	Mouse monoclonal	1:300	No	Abcam Inc Cambridge, MA

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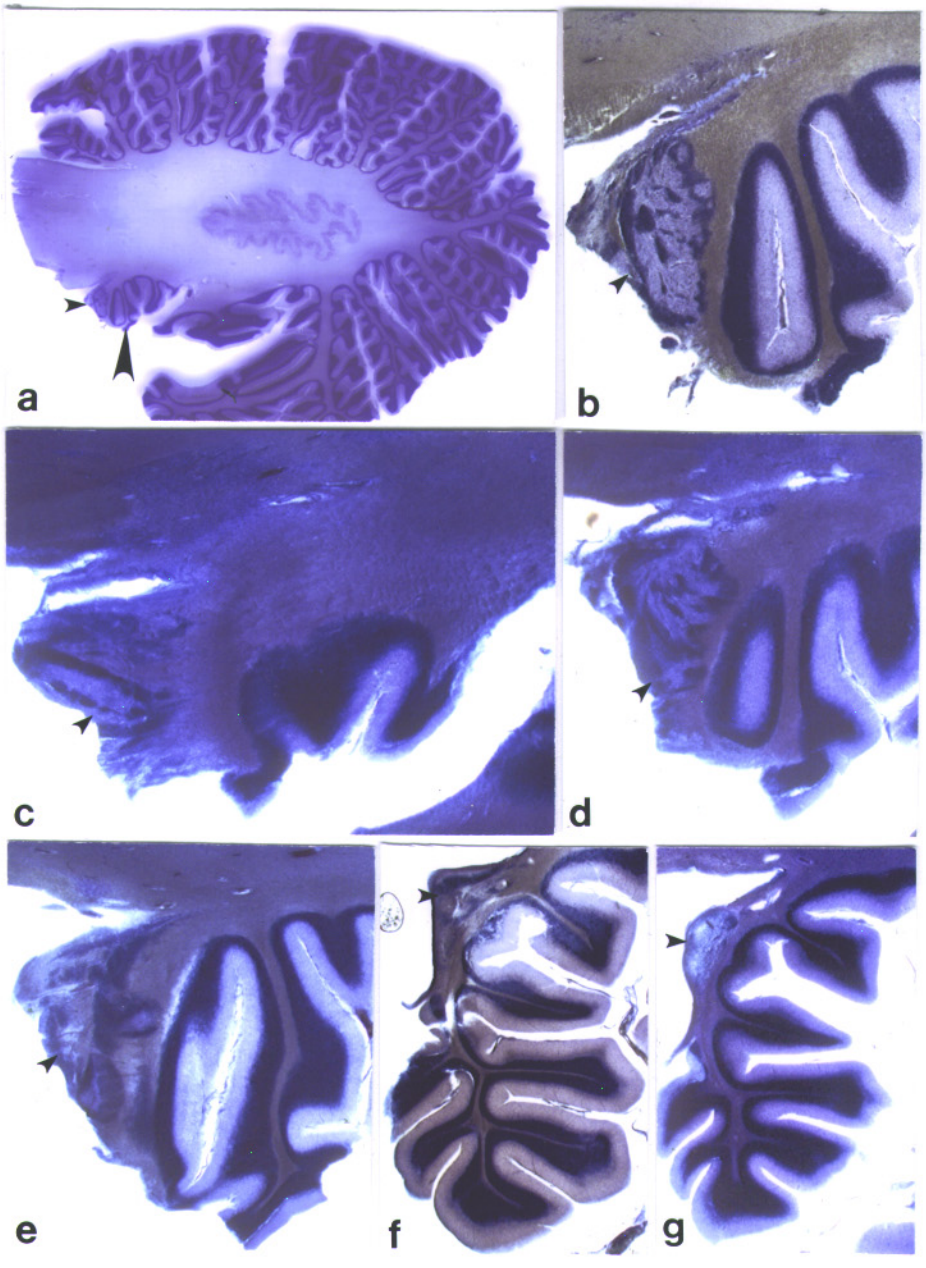
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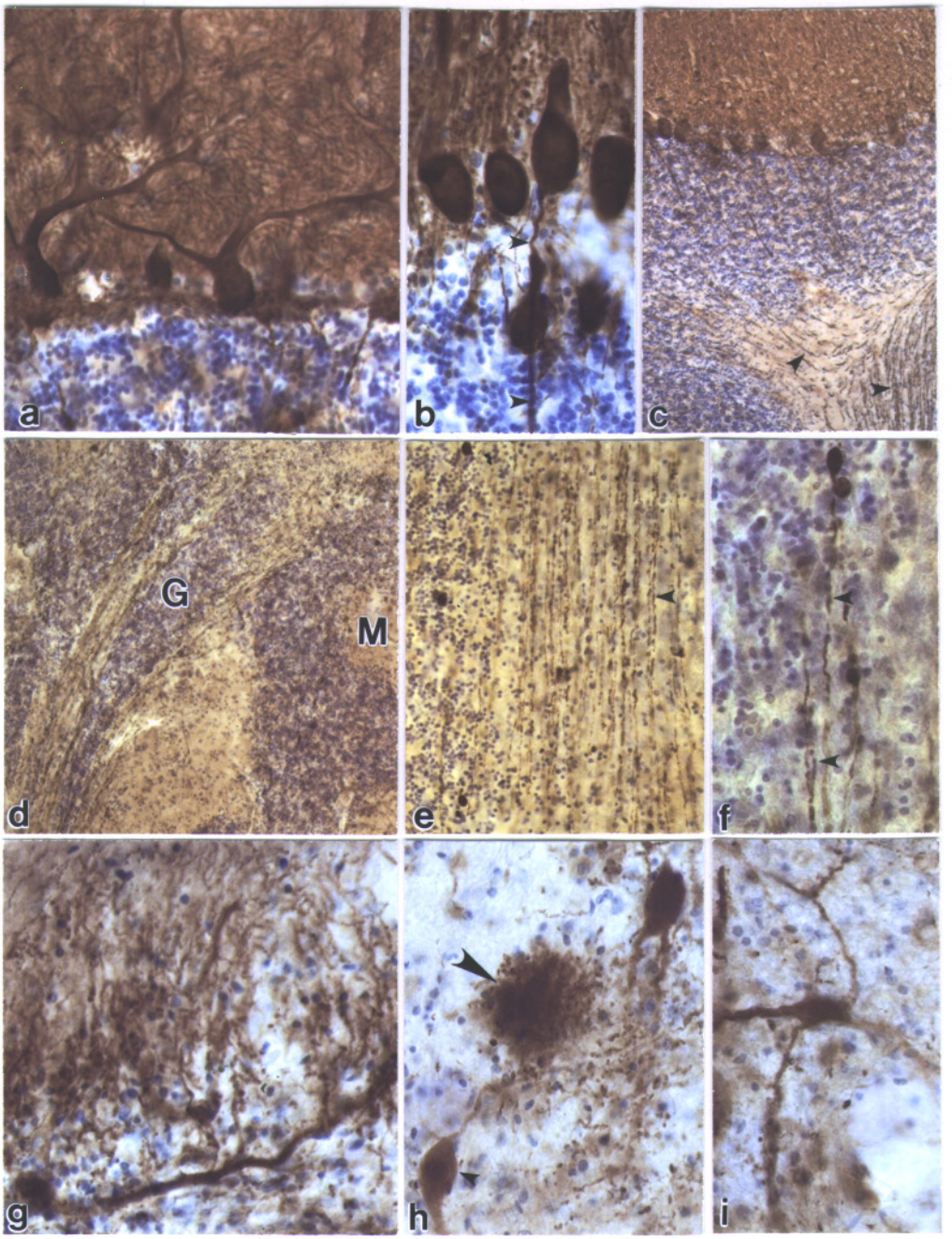
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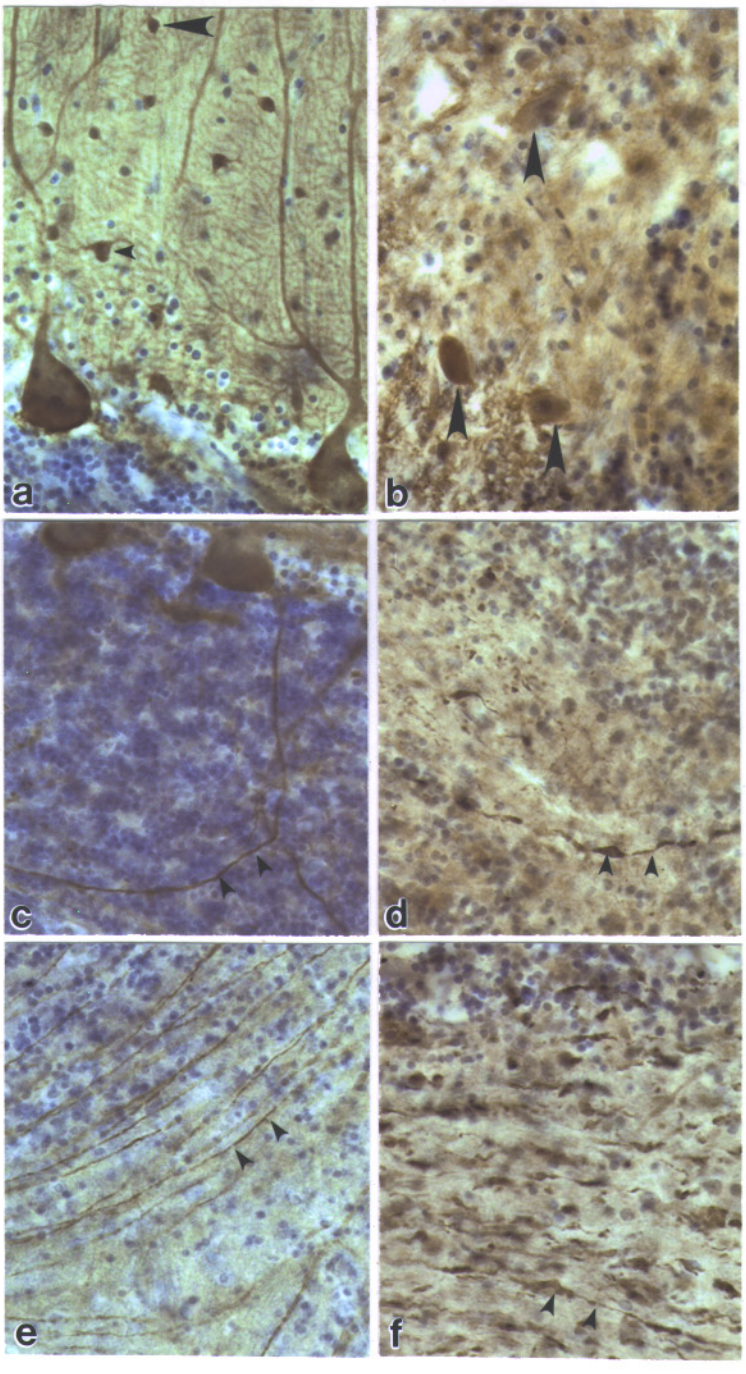
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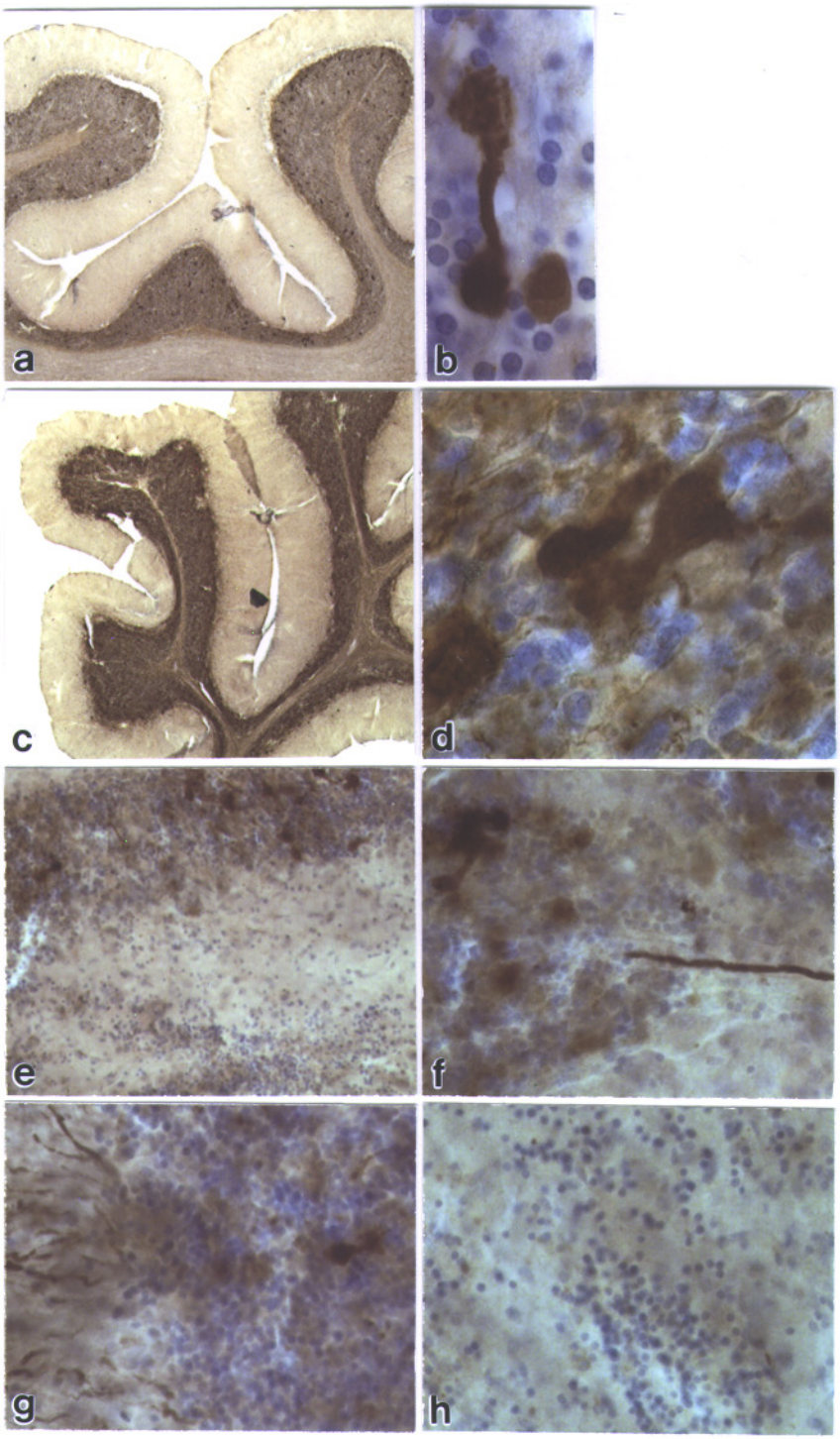
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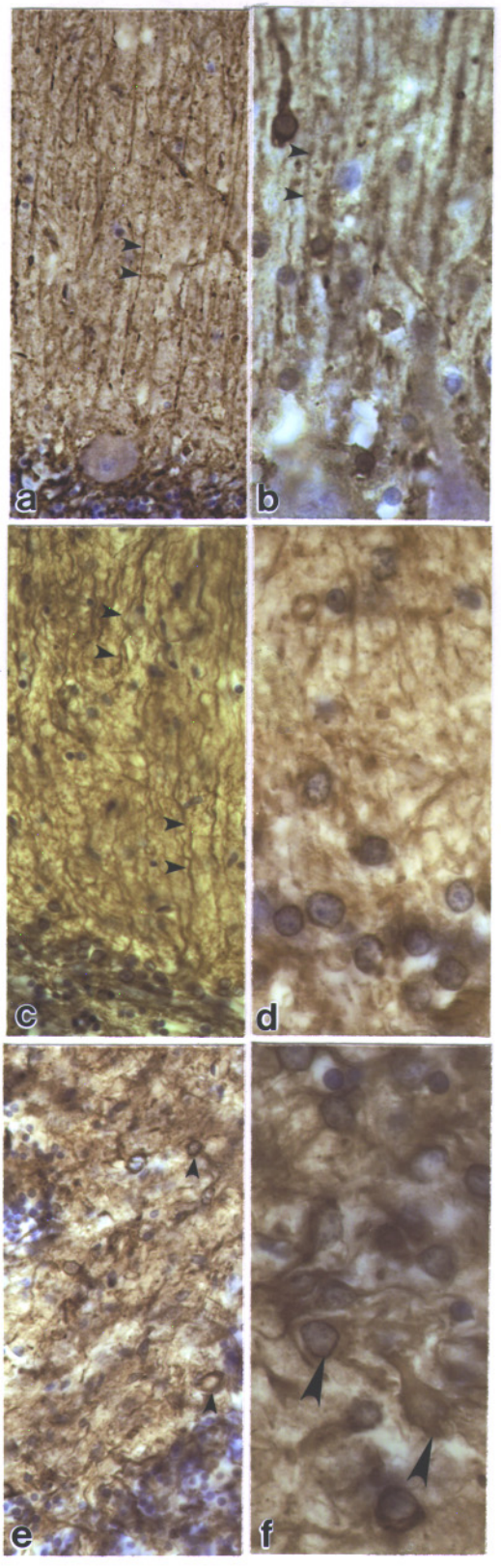
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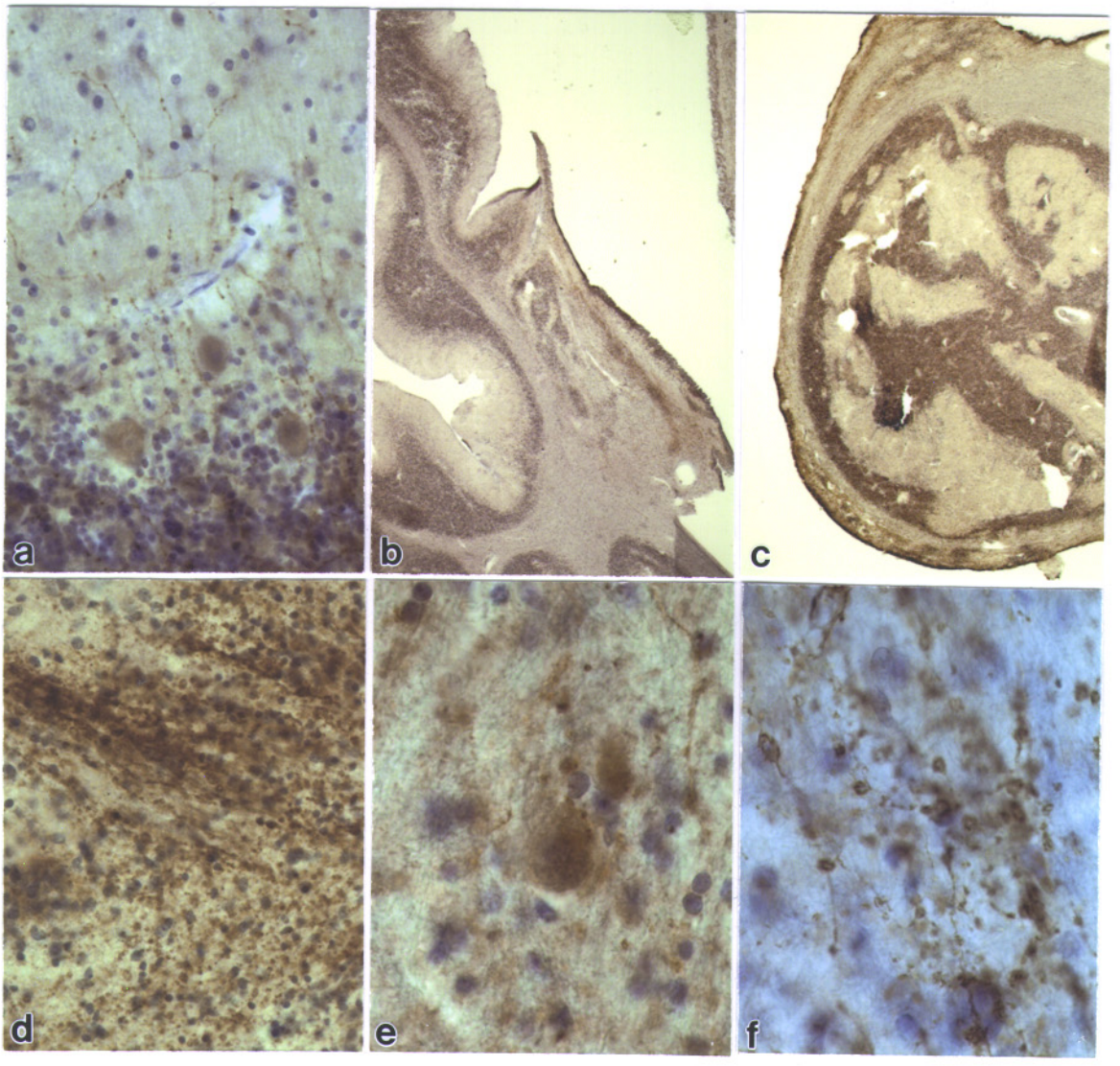
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Clinicopathological Stratification of Idiopathic Autism and Autism Associated with Duplications 15q11.2-q13

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Abstract

Postmortem studies of brains of individuals with idiopathic autism and duplications 15q11.2-q13 autism identify a cluster of neuropathological features differentiating these cohorts. They show a need for both sub-classification of autism according to etiology, clinical presentation, and neuropathology, and a commonality of clinical and neuropathological traits justifying autism diagnosis. The features differentiating these cohorts include: (a) maternal origin dup(15), (b) autism in 78% of subjects, (c) more severe clinical phenotypes, with intellectual deficit (100%), early-onset of severe or intractable seizures in 78% of subjects, and increased to 67% prevalence of sudden unexplained death, (d) high prevalence of microcephaly, with mean brain weight 300g less than in idiopathic autism, (e) several-fold increase in the number of developmental abnormalities, including defects of migration and dysplastic changes, especially numerous in the hippocampal formation, and (f) significant increase of the intraneuronal amyloid load, reflecting enhanced amyloid-β precursor protein processing with α-secretase.

Key Words: Autism, Chromosome 15q11.2-q13 duplication, Brain, Development, Heterotopia, Dysplasia, Amyloid beta, Epilepsy, Sudden unexpected death.

Genetic factors in autism. In 1977 Folstein and Rutter (Folstein and Rutter, 1977) demonstrated a striking difference in concordance rates of autism between monozygous and dizygous twins. The studies that followed revealed close to 90% monozygous concordance rates for autism spectrum disorder (ASD) and very low concordance rates for dizygotic twins (Bailey *et al*., 1995), showing a significant role of genetic factors in autism etiology (Ritvo *et al.,* 1985; Smalley *et al.*, 1988, Steffenburg *et al*., 1989; Folstein and Piven, 1991; Rutter *et al.*, 1990a,b; Lotspeich and Ciaranello, 1993). Recent studies demonstrate contribution of both genetic and environmental factors to autism and ASD. Liu *et al*. (2010) revealed 57.0% and 67.2% concordance rates for monozygotic males and females, respectively, 32.9% concordance rates for same sex dizygotic twins, and 9.7% recurrence risk for siblings, whereas Hallmayer *et al*. (2011) demonstrated moderate, 37% and 38% genetic heritability for autism and ASD, respectively, and 55% contribution of shared environmental factors to autism and 55% to ASD.

Genetic basis has been revealed for less than 15% of autism cases, whereas no single genetic cause explains more than 2% (Abrahams and Geshwind 2008, Wang *et al*., 2009). Chromosomal abnormalities, especially large chromosomal anomalies, such as unbalanced translocations, inversions, rings, and interstitial deletions and duplications, were detected in 1.7% to 4.8% of subjects diagnosed with ASDs (Lauritsen *et al*., 1999; Wassink *et al*., 2004). They are identified as duplications of 15q [dup(15)], deletions of 18q, Xp, 2q, and such sex chromosome aneuploidies as 47,XYY and 45,X (Gillberg, 1998; Reddy, 2005). Autism is diagnosed in 69% of subjects with maternal origin duplications 15q11.2-q13 (dup15) (Rineer *et al*., 1998), in 15% to 28% of individuals with fragile X syndrome (FXS) (Hagerman, 2002), and in 7% of people with Down syndrome (DS) (Kent *et al*., 1999).

Duplications of chromosome 15q11q13. The imprinted chromosome region 15q11q13 is known for its instability, resulting in the DNA repeats or deletions associated with several syndromes. Prader Willi syndrome (PWS) is predominantly the result of a paternal deletion of the small nuclear ribonucleoprotein polypeptide N (SNRPN) gene in 15q11q13 (Ozcelik *et al*., 1992), whereas the Angelman syndrome (AS) is most often a result of maternal deletion of the ubiquitin-protein ligase E3A (UBE3A) gene (Knoll *et al*., 1993). Subsets of subjects with PWS or AS have been reported to exhibit autistic-like behavior (Arrieta *et al*., 1994; Demb and Papola, 1995; Dykens and Kasari, 1997; Penner *et al*., 1993; Steffenburg *et al*., 1996; Summers *et al*., 1995). Chromosomal abnormalities of the proximal 15q region belong to the most common genomic aberrations detected in autistic disorder

probands (Arrieta *et al*., 1994; Baker *et al*., 1994; Bundey *et al*., 1994; Cook EH *et al*., 1997; Flejter *et al*., 1996; Gillberg *et al*., 1991; Hotopf and Bolton, 1995; Kerbeshian *et al*., 1990; Martinsson *et al*., 1996; Schroer *et al*., 1998; Weidmer-Mikhail *et al*., 1998; Wolpert *et al*., 2000a,b). These abnormalities were found in up to 3% of subjects diagnosed with autism. An especially strong association with autism was revealed in duplications in the range of 8 to 12 Mb derived from the maternal chromosome (Cook EH *et al*., 1997; Dawson *et al*., 2002). Interstitial triplications [int trp(15)] are relatively rare but have invariably been associated with a severe phenotype, including intellectual deficit (ID), ASD, and seizures. A few subjects have been diagnosed with duplications of paternal origin; however, they were described as clinically unaffected (Bolton *et al*., 2001; Cook EH *et al*., 1997; Mohandas *et al*., 1999; Schroer *et al*., 1998) or affected but without ASD (Mao *et al*., 2000; Mohandas *et al*., 1999). In only one subject was paternal origin dup(15) associated with ASD (Bolton *et al*., 2004). Because only maternally inherited aberrations of chromosome 15q11q13 have been reported to be associated with severe clinical phenotype, one may assume that the copy number of maternal genes within this genomic region contributes to alterations of brain development and the autistic phenotype.

Gene expression in dup(15). Postmortem studies of the brain reveal that chromosome duplications 15q11-13 are associated with epigenetic alterations in gene expression that are not predicted from copy number (Hogart *et al*., 2008). Whole-genome expression profiling of lymphoblast cell lines derived from individuals with autism and isodicentric 15 [idic(15)] revealed 112 transcripts that are significantly dysregulated in samples from subjects with duplications. However, only four of a total of 80 genes located within the duplicated area of chromosome15 were found to be up-regulated, including 1.5- to 2.0-fold up-regulation of ubiquitin protein ligase E3A (*UBE3A*; 15q11-q13) and 1.89-fold increase of *HERC2*. Baron *et al.* (2006) concluded that the majority of changes are not due to increased gene dosage in a critical chromosome 15 region, but represent potential down-stream effects of this duplication, including two down-regulated genes: *APP* encoded by a gene on chromosome 21, and *SUMO1* encoded by a gene on chromosome 2. Several functional categories were identified as associated with macromolecular catabolic processes, including the ubiquitin-dependent protein catabolism. The increase of UBE3A protein level may indicate dysregulation of ubiquitin-mediated proteasome pathway in cells with dup(15), resulting in enhanced ubiquitination of proteins for non-lysosomal degradation/disposal in response to genotoxic insult. Down-regulation of SUMO1, a

ubiquitin-like molecule, may indicate other forms of dysregulation of cell catabolic processes leading to decreased cell sensitivity to apoptotic stimuli and tolerance to DNA damage (Baron *et al*., 2006).

The γ-aminobutyric acid type$_A$ (*GABA$_A$*) receptor subunit genes (*α5*, *β3,* and *γ3*) are located in the susceptibility segment of duplicated chromosome 15 (Bass *et al*., 2000; Buxbaum *et al*., 2002; Cook *et al*., 1998; Menold *et al*., 2001). GABA is the main inhibitory neurotransmitter binding to a complex of GABA$_A$ receptors. Polymorphisms in the *GABA$_A$-β3* receptor subunit are associated with autism (Cook *et al*., 1998; Martin *et al*., 2000). Moreover, differential methylations in the *GABA$_A$* gene (Bittel *et al*., 2005; Gabriel *et al*., 1998; Hogart *et al*., 2007; Meguro *et al*., 1997;) may result in epigenetic modifications and modifications of clinical phenotypes in dup(15)/autism.

Clinical characteristics. Between 1994 and 2008, approximately 160 patients diagnosed with inverted (inv) dup(15) were characterized (Battaglia, 2008; Dennis *et al*., 2006; Wang *et al*., 2004; Webb, 1994; Webb *et al*., 1998; Wolpert *et al*., 2000a,b;). A similar male:female ratio (3:1) has been reported in idiopathic autism (Bryson, 1996) and in probands with dup (15q) (Wolpert *et al*., 2000a).

Clinicopathological correlations in dup(15) cohorts with considerable variations in the breakpoints, copy numbers, additional genetic and epigenetic modifications, and significant clinical differences are limited. In spite of these differences, distinctive clinical features of dup(15) syndrome, including early central hypotonia, developmental delay, intellectual disability, epilepsy, and autistic behavior, were defined. Battaglia's summary of clinical findings (2008) suggests that (a) in more than 75% of individuals with dup(15) hypotonia and lax ligaments, developmental delay and intellectual disability, autistic behavior, epilepsy, and minor dysmorphic features, involving mainly the face, are present; (b) from 25% to 50% of subjects are affected with brain abnormalities, growth retardation, and gastro-urinary tract defects; and (c) in less than 25% of subjects, microcephaly and congenital heart defects are observed. Wolpert *et al.* (2000a) documented that multiple maternal copies of the proximal 15q region may lead to one form of autistic disorder involving genes in the 15q11q13 region. Social, communicative, and behavioral function, and many clinical features are similar among individuals with autism associated with dup(15) and those with autism arising from other causes. The authors' review shows a much greater occurrence rate of seizures in dup(15) (69%) than reported in idiopathic autism (33%) (Tuchman and Rapin, 2002; Tuchman *et al*., 2009), a common delay in achieving motor milestones (77%), and hypotonia (77%) in dup(15) and a much lower occurrence rate of these abnormalities in idiopathic autism. Almost all individuals with inv dup(15) are affected with moderate to profound developmental delay and intellectual disability (Battaglia *et al*., 1997; Battaglia, 2008;

Crolla *et al*., 1995; Gillberg *et al*., 1991; Webb, 1994; Webb *et al*., 1998; Robinson *et al*., 1993; Wolpert *et al*., 2000a).

Earlier studies of smaller groups of patients based on non-standardized criteria revealed autism in 33% (Leana-Cox *et al*., 1994) and 36% (Crolla *et al*., 1995) of individuals diagnosed with syndrome. Application of standardized assessment of autistic symptoms to a cohort of 29 children and adults with dup(15) revealed that 69% of these subjects fulfilled the criteria of autism diagnosis (Rineer *et al*., 1998).

Neuropathology of autism with dup(15) and with idiopathic autism. The current knowledge of brain developmental alterations is based on results of examination of brains of nine individuals diagnosed with dup(15), including seven subjects (78%) diagnosed with autism (Wegiel *et al.*, 2012a,b). These studies demonstrate several striking differences and some similarities between subjects diagnosed with autism associated with dup(15) and idiopathic autism. These patterns indicate that autistic phenotype might be a product of etiologically, qualitatively, and quantitatively different processes.

Increased prevalence of brain transient overgrowth and macrocephaly in idiopathic autism and microcephaly in dup(15) autism. Macrocephaly, defined as head circumference greater than the 97th percentile of the normal population, has been reported in more than 20% of autistic subjects (Bailey *et al*., 1995; Bolton *et al*., 1994; Fombonne *et al*., 1999; Lainhart *et al*., 1997). Increased brain weight was also reported in autism in postmortem studies (Bailey *et al*., 1993; Bauman and Kemper, 1985). A short period of increased brain size starting at the age of less than 1 year (Courchesne *et al*., 2003; Lainhart *et al*., 1997; Redcay and Courchesne, 2005) results in macrocephaly in 37% of autistic children under the age of 4 years (Courchesne *et al*., 2001) and macrocephaly in 42% of the 19 twins diagnosed with idiopathic autism under the age of 16 years (Bailey *et al*., 1995). Brain overgrowth is associated with increased number of neurons (Courchesne *et al*., 2011). However, approximately 15% of 2- to 16-year-old autistic children are affected by microcephaly (Fombone *et al*., 1999), which is associated frequently with a more severe clinical phenotype (Guerin *et al*., 1996; Hof *et al*., 1991), including ID and other disorders (Fombone *et al*., 1999).

The characteristics of individuals with dup(15) are few, but published reports show opposite proportions between macrocephalic and microcephalic subjects, unlike in idiopathic autism. In the

largest examined cohort (n = 107) with dup(15), macrocephaly was detected in only 2.8%, and microcephaly in six times (16.8%) more subjects (Schroer *et al*., 1998). The first postmortem study of the brains of nine subjects diagnosed with dup(15), including seven subjects diagnosed with autism (78%) revealed mean brain weight 303 g less than in the idiopathic autism group ($p < 0.001$) (Wegiel *et al* 2012 a,b). Age-adjusted mean brain weight for dup(15) (n = 9), idiopathic autism (n = 10) and control (n = 7) subjects was 1,171g, 1474 g, and 1378 g, respectively. The difference between the dup(15) and control group was non-significant but suggestive ($p = 0.06$) (Fig. 1).

Two postmortem studies of idiopathic autism cohorts revealed epilepsy in six per 13 subjects (46%; Wegiel *et al*., 2010) and 3/10 (30%), whereas in the dup(15) group, epilepsy was diagnosed in seven of nine cases (78%) (Wegiel *et al*., 2012a). In the dup(15) cohort, sudden and unexplained death in patients with epilepsy (SUDEP) was diagnosed in six of nine subjects (67%), and seizure-related death was determined in one case (10%), resulting in 77% of cases of sudden death (Wegiel *et al*., 2012a). Sudden death in the idiopathic autism cohort was reported in four of 13 subjects (31%, Wegiel *et al*., 2010). These data suggest that the microcephaly and very early onset of seizures are risk factors for SUDEP in the subpopulation of dup(15) subjects, and that this risk is at least two times greater in dup(15) than in idiopathic autism.

Neuropathological stratification of developmental abnormalities in dup(15) and idiopathic autism cohorts. Applications of an extended neuropathological protocol based on examination of approximately 120 serial hemispheric sections per brain resulted in the detection of each developmental abnormality larger than 2–3 mm. Three major types of developmental changes were detected, including heterotopias, dysplastic changes, and abnormal neuronal proliferation. They were found in all nine dup(15) and all 10 subjects with idiopathic autism; however, the type, topography, and number of abnormalities show significant differences between these two cohorts (Fig. 2).

Heterotopias. Defects of migration resulting in heterotopias in the alveus, CA4, and dentate gyrus (DG) occur very often in the dup(15) group (89%), are rare in individuals with idiopathic autism (10%, $p = 0.001$), and are not present in control subjects. However, the prevalence of the heterotopias in cerebellar white matter is comparable in dup(15) (56%) and idiopathic autism (60%). The heterotopias in cerebral white matter are rare in both cohorts (11% and 10%, respectively). These three patterns illustrate not only striking topographical differences in the distribution of defects of neuronal migration but also significant differences between idiopathic autism and autism associated with dup(15).

Dysplasia. Microdysgenesis resulting in focal developmental alterations of cytoarchitecture is also topographically selective and is detected mainly in the DG and cornu Ammonis in the hippocampal formation, and in the cerebral and cerebellar cortex. Dysplastic changes in the DG have been identified as hyperconvolution of the DG, duplication of the granular layer, massive protrusions of the granular layer into the molecular layer, focal thickening, thinning and fragmentation of the granular layer. The prevalence of dysplasia in the DG is several times higher (89%) in subjects with dup(15) than in the idiopathic autism group (10%; $p < 0.001$) (Fig. 3). In the DG, usually only one type of these changes is observed in idiopathic autism, whereas from two to five types are observed in each brain in dup(15) autism. However, dysplastic changes in the cornu Ammonis are rare in both cohorts, and the percentage of affected subjects is comparable: 20% in dup(15) autism and 22% in idiopathic autism.

Another feature distinguishing these two cohorts is cerebral cortex dysplasia, which is detected in 50% of subjects with dup(15) but is absent in idiopathic autism and in control subjects. The diversity of neocortical dysplastic changes, including multifocal cortical dysplasia with focal hypo- or acellularity, loss of vertical and horizontal organization, focal polymicrogyria and bottom-of-a-sulcus dysplasia, suggests that cortical abnormalities in dup(15) autism are the product of the distortion of several different mechanisms of cortex development.

The subependymal nodular dysplasia detected in the lateral ventricle in the occipital lobe in the brain of 15- and 39-year-old females diagnosed with dup(15) autism, and in 7- and 32-year-old males diagnosed with idiopathic autism are evidence of abnormal neuronal proliferation in some autistic subjects regardless of autism etiology and identify the predilection site for this developmental defect, which is detectable also on MRI scans (Wegiel *et al*., 2010, 2012a).

Causative link between developmental neuropathological changes, epilepsy, and sudden death in childhood. Similar hippocampal developmental abnormalities observed in sudden unexpected and unexplained death in childhood (SUDC) cases (Kinney *et al*., 2007) are considered an epileptogenic focus that might be triggered by infection, fever, or head trauma and result in seizures and unwitnessed death (Blum *et al*., 2000; Frysinger and Harper, 1990; Yang *et al*., 2001). SUDC in two subjects and SUDEP in five cases with dup(15) and autism and several-fold higher prevalence of hippocampal and cortical dysplasia than in idiopathic autism appears to be the clinicopathological criterion for stratification between and within these cohorts. These developmental alterations are not pathognomonic of an “epileptic brain” (Kinney *et al*., 2007), but the combination of microcephaly, the

2.5-fold higher prevalence of several types of developmental abnormalities, the 2.3-fold higher prevalence of epilepsy, and the six-times higher prevalence of epilepsy-related death in the dup(15) cohort suggests that unique genetic and molecular modifications and developmental structural defects distinguish these two cohorts of autistic subjects.

The link between dysplastic changes in the cerebellar flocculus and atypical gaze. Cerebellar abnormalities are among the most consistent developmental alterations detected in autism (Bauman and Kemper, 1996; Courchesne *et al*., 2001; Kemper and Bauman, 1993; Ritvo *et al*., 1986; Whitney *et al*., 2008, 2009). A reduced number of Purkinje cells (PCs) has been detected in 72% of the reported autism cases (Palmen *et al*. review, 2004), but studies by Whitney *et al*. suggest that the reduced number of PCs is not the effect of developmental deficit, but is instead t he result of early neuronal loss (Whitney *et al*., 2008, 2009). The prevalence of defective migration of cortical neurons and dentate nucleus neurons in the cerebellar white matter was almost identical in dup(15) autism and idiopathic autism (56% and 60%, respectively). Four types of cerebellar dysplasia, including nodulus, flocculus, and vermis dysplasia, and focal polymicrogyria, were found in dup(15) autism and idiopathic autism. Vermis dysplasia and focal polymicrogyria were rare in both groups.

However, a portion of the flocculus and a small portion of the nodulus, developmentally related to the flocculus (flocculus-like" region of the ventral paraflocculus; Tan *et al*., 1995), were affected by dysplastic changes. Flocculus dysplasia is associated with a striking deficit of PCs and unipolar brush cells, an almost complete lack of inhibitory basket and stellate cells in the molecular layer, and reduction of the number of granule cells. This abnormal flocculus cytoarchitecture appears to be an indicator of the profound disruption of the olivo-floccular circuitry and severe functional alterations. The flocculus participates in the control of eye motion (Leung *et al*., 2000), and coordination of eye and head movements during active gaze shifts by modulating the vestibulo-ocular reflex (Belton and McCrea, 1999). Dysplastic changes are observed in the nodulus of autistic and control subjects, but the function of the affected region of the nodulus is not clear. The presence of dysplasia in the flocculus in 75% of individuals with dup(15) autism (Wegiel *et al*., 2012b), in 50% and 67% of idiopathic autism cases (Wegiel *et al*., 2012a,c), and 20% of control subjects (Wegiel *et al*., 2012c) and the presence of olivary dysplasia in three of five autistic subjects and ectopic neurons related to the olivary complex in two cases reported by Bailey *et al*. (1998) indicate that both major structural and functional components of the olivo-floccular circuitry are prone to developmental defects, most likely contributing to the atypical gaze of autistic subjects. These findings also suggest that flocculus

developmental defects are observed in autistic subjects regardless of etiology. Individuals diagnosed with idiopathic autism and dup(15) autism reveal altered oculomotor functions, including atypical gaze, impairments in smooth pursuit, and deficits in facial perceptions, suggesting defects in the olivo-floccular neuronal circuit. These defects are reported early in the development of children with autism (Mundy *et al*., 1986; Dawson *et al*., 1998) and contribute to deficits in using gaze to understand the intentions of other people and their mental states (Baron-Cohen, 1995; Baron-Cohen *et al*., 1999, 2001; Leekam *et al*., 1998, 2000).

Increased levels of secreted amyloid precursor protein-alpha (sAPP) and reduced levels of Aβ40 and Aβ42 in the blood plasma. Significantly lower concentrations of both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, and a reduced ratio of $A\beta_{40/42}$ detected in the blood plasma of 52 autistic children 3 to 16 years of age compared to 39 age-matched control subjects were attributed to the loss of Aβ equilibrium between the brain and blood (Al-Ayadhi *et al*., 2012). Significantly increased levels of sAPP-α in blood plasma in 60% of autistic children (Sokol *et al.*, 2006) indicate enhanced non-amyloidogenic APP processing by α-secretase. Higher levels of sAPP-α in blood plasma were especially prominent in autistic subjects with aggressive behavior (Ray *et al*., 2011; Sokol *et al*., 2006). Enhanced non-amyloidogenic cleavage of APP with α-secretase is associated not only with autism (Bailey *et al*., 2008; Ray *et al*., 2011; Sokol *et al*., 2006; Sokol *et al*., 2011; Wegiel *et al*., 2012b), but also with FXS (Sokol *et al*., 2011; Westmark and Malter, 2007; Westmark *et al*., 2011). Due to the neurotrophic activity of sAPP-α, increased levels of this APP metabolite are considered a co-factor contributing to brain overgrowth in autism and FXS (Sokol *et al*., 2011). The fragile X mental retardation protein (FMRP) binds to and represses translation of APP mRNA. The absence of FMRP in people diagnosed with FXS results in upregulation of APP, $A\beta_{40}$, and $A\beta_{42}$. Similar upregulation is detected in $Fmr1^{KO}$ mice (Westmark and Malter, 2007); however, genetic reduction of *AβPP* by removal of one *App* allele in $Fmr1^{KO}$ mice reverses the FXS phenotype and increases blood plasma levels of $A\beta_{1\text{-}42}$ to control levels (Westmark *et al*., 2011).

Enhanced accumulation of amino-terminally truncated Aβ in neuronal cytoplasm. Neuronal proteolytic cleavage of APP by β- and γ-secretases (amyloidogenic pathway) results in release of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, which are able to form fibrillar deposits in the extracellular space (amyloid plaques) and in the wall of brain vessels (amyloid angiopathy). $A\beta_{17\text{-}40}$ and $A\beta_{17\text{-}42}$ is a product of α- and γ-secretases (p3 peptide) in the non-amyloidogenic pathway (Iversen *et al*. 1995; Selkoe, 2001). Aβ is generated and detected in the endoplasmic reticulum/Golgi apparatus and endosomal-lysosomal pathway (Cook D.G. *et al*., 1997; Glabe, 2001; Greenfield *et al*., 1999; Hartmann *et al*., 1997; Wilson *et al*., 1999),

multivesicular bodies (Takahashi *et al*., 2002), and mitochondria (Bayer and Wirths, 2010; Casperson *et al*., 2005). Aβ peptides differ in oligomerization and fibrillization as well as toxicity. Intraneuronal accumulation has been reported in normal human brain (Wegiel *et al*., 2007). Enhanced accumulation has been proposed as an early alteration in Alzheimer's disease (AD) and in transgenic mouse models of AD (Bayer and Wirths, 2010; Gouras *et al*., 2010; Gyure *et al*., 2001; Mochizuki *et al*., 2000; Winton *et al*., 2011).

Intraneuronal Aβ in human brain is mainly amino-terminally truncated $A\beta_{17-40}$ and $A\beta_{17-42}$ (Wegiel *et al*., 2007). Cytoplasmic Aβ in neurons is a reflection of the balance between its rate of synthesis, accumulation in cytoplasmic organelles, and degradation. The extracellular level of Aβ is a reflection of neuronal production and extracellular oligomerization, fibrilization, deposition, and disposal, including drawing of the excessive amounts through the blood-brain barrier to the blood (Weller *et al*., 1998). The morphology and amount of intracellular deposits of Aβ are neuron-type–specific and show a broad spectrum of differences in developing and aging brains and in brains affected by pathology. Increased level of sAPP-α in the blood plasma of autistic subjects is linked to enhanced intraneuronal accumulation of amino-terminally truncated $A\beta_{17-40/42}$ in the neurons of autistic subjects (Wegiel *et al*., 2012b).

Stratification of Aβ accumulation in neurons in the dup(15) autism and idiopathic autism. Postmortem study of 12 brain structures and neuronal populations (frontal, temporal, and occipital cortex; amygdala, thalamus, lateral geniculate body, DG; CA1 and CA4 sectors and dentate nucleus in the hippocampal formation; and PCs) revealed higher Aβ load in neurons in 11 subregions in dup(15) autism than in idiopathic autism ($p < 0.0001$) and in control subjects ($p < 0.0001$). In eight regions, cytoplasmic Aβ load was significantly higher in idiopathic autism than in control subjects ($p < 0.001$).

Excessive accumulation of Aβ in two autistic cohorts was neuron-type–specific. Classification of neuronal Aβ immunoreactivity as strong, moderate, and weak revealed two types of alterations. Type 1 is characterized by a significant increase in the percentage of neurons with strong Aβ immune-reactivity, defined as a condensed mass of indistinguishable small and large immunoreactive granules occupying a large portion of neuron perikaryon. Type 1 of Aβ accumulation is typical for the amygdala, thalamus, and lateral geniculate body (LGB) (Fig. 4). Stratification of dup(15)autism and idiopathic autism cohorts is reflected in a 7.6-fold increase of the percentage of strongly positive neurons in the amygdala and thalamus and a 4.5-fold increase in the LGB in dup(15) autism in comparison to the control cohort. In idiopathic autism, the increase was 5.3x, 6.3x, and 3.9x,

respectively, in comparison to in the control subjects. Type 2 of Aβ intraneuronal accumulation is distinguished by a relatively low percentage of neurons with strong Aβ immunoreactivity, but a higher total percentage of Aβ-positive neurons. This pattern was typical for pyramidal neurons in all three examined neocortical regions (frontal, temporal, and occipital cortex), and the total percentage of Aβ-positive neurons was higher in dup(15) than in the autism and control groups ($p < 0.001$).

$A\beta_{1-40}$ and $A\beta_{1-42}$ in diffuse plaques in autism. Diffuse amorphous nonfibrillar Aβ deposits are classified as preplaques (Mann *et al*., 1989) or pre-amyloid deposits (Tagliavini *et al*., 1989); however, diffuse deposits in human cerebellar cortex and the parvocellular layer of the presubiculum do not fibrilize, regardless of age or stage of AD (Wisniewski *et al*., 1998). Diffuse plaques were found in two of the 11 subjects diagnosed with ASD (51 and 52 years old) and in one of nine subjects diagnosed with dup(15) and autism (Fig. 5). These subjects were the oldest in both examined groups. Plaques were nonfibrillar, thioflavin S–negative, but in contrast to amino-terminally truncated $A\beta_{17-40/42}$ in neurons, they contained full-length $A\beta_{1-40/42}$ (Wegiel *et al*., 2012b). Diffuse plaques are also observed in young people diagnosed with DS, but they are $A\beta_{17-40/42}$-positive (Gowing *et al*., 1994;, Lalowski *et al*., 1996).

Both in the plaque perimeter and in plaque-free areas, numerous astrocytes and some microglial cells contain amyloid, but only amino-terminally truncated $A\beta_{17-40/42}$. Focal enhanced proliferation of astrocytes, accumulation of Aβ in their cytoplasm, and accelerated death results in Aβ deposition, mainly in the perivascular space.

These findings suggest that the pattern of metabolic alterations of APP processing and Aβ accumulation is comparable in two autistic cohorts, but the severity of metabolic changes is significantly intensified in dup(15) autism in comparison to idiopathic autism. Enhanced APP processing with α- and γ-secretase increases the percentage of Aß-positive neurons and intracellular amyloid load in dup(15) autism with microcephaly. Increased levels of blood plasma neurotrophic sAPP-α detected in 60% of subjects with idiopathic autism justified the hypothesis that the increased level of the product of α-secretase may help identify a subset of children in which early brain overgrowth is sufficient for development of autism and might be a marker of the mechanism that regulates brain overgrowth (Sokol *et al*., 2011). One may hypothesize that in subjects with dup(15), genetic factors contribute to microcephaly and dominate over metabolic modifications and elevated levels of neurotrophic products of APP processing.

Closing remarks. Comparative postmortem studies of the brains of individuals diagnosed with dup(15) autism identify a cluster of neuropathological findings differentiating this cohort of autistic subjects with genetically identified autism etiology from a cohort of subjects with idiopathic autism. These studies support the recommendation by Happe *et al*. (2006) for fractionation of autism into "autisms" with different etiologies, clinical presentations, and neuropathology, and most likely requiring different preventive strategies and different treatments.

Because the complex nature of developmental abnormalities increases the risk of death in childhood and early adulthood in the dup(15) autism cohort, postmortem study appears to reflect both developmental abnormalities associated with this genetic trait, and a particular combination of factors contributing to early death. The list of factors defining the risk of early death and the detected pattern of neuropathological changes includes: (a) maternal origin dup(15), (b) autism, (c) more severe clinical phenotypes with ID, early-onset and severe or intractable seizures, and increased prevalence of SUDEP, (d) high prevalence of microcephaly, (e) several-fold increase in the number of developmental abnormalities, including defects of migration and multifocal defects of cytoarchitecture, especially numerous in the hippocampal formation, and (f) several-fold increase in the percentage of neurons with increased amyloid load, reflecting enhanced APP processing in non-amyloidogenic pathway with α- and γ-secretase ($A\beta_{17\text{-}40/42}$); enhanced proliferation and activation of $A\beta_{17\text{-}40/42}$-positive astrocytes, enhanced rate of astrocytes death, and in some cases, an early onset of diffuse plaques containing $A\beta_{1\text{-}40/42}$.

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Figure legend

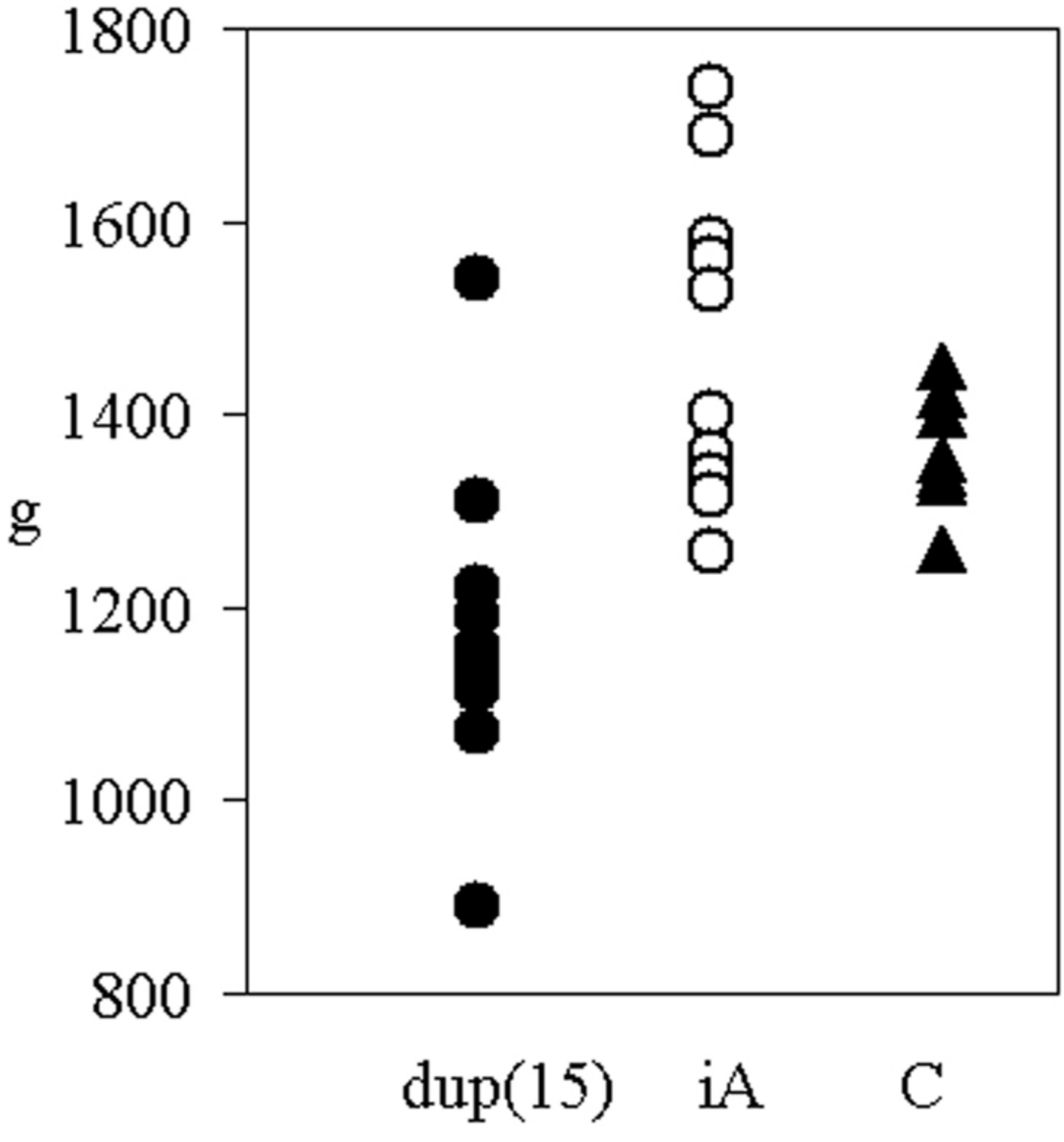
Figure 1. Significant reduction of mean brain weight (g) in the dup(15) autism group (1,171 g) in comparison to the idiopathic autism (iA) cohort (1,474 g; $p < 0.001$), and insignificant but suggestive reduction in comparison to the control (C) group (1,378 g; $p < 0.06$).

Figure 2. The mean number of developmental alterations detected in postmortem evaluation of serial hemispheric sections was 2.5x higher in the dup(15) autism group (7.1/case) than in the idiopathic autism (iA) group (2.8/case).

Figure 3. Topography and morphology of 11 types of developmental abnormalities in the brain of a 24-year–old male diagnosed with dup(15), autism, severe seizures, and SUDEP: dentate gyrus (DG) hyperconvolution and DG heterotopia in the CA4 (a); DG granule cell layer protrusion (b; arrowhead), duplication (c), focal thinning and discontinuity (d), granule cell layer fragmentation (e; arrowhead); multifocal microdysgenesis within CA4 (e, two arrowheads); larger magnification of different types of microdysgenesis within CA4 (f, g); cerebellar heterotopia with morphology of cerebellar deep nuclei (h, i) and with modified morphology of cerebellar cortex (j, k); and dysplasia in the cerebellar nodulus (l) and flocculus (m).

Figure 4. The percentage of neurons with very high intracellular Aβ load is significantly higher in the amygdala, thalamus, and Purkinje cells in dup(15) autism than idiopathic autism and control subjects ($p < 0.0001$). In the frontal, temporal, and occipital cortex, the total percentage of Aβ-positive neurons (neurons with strong, moderate, and weak Aβ immunoreactivity) is significantly higher in dup(15) autism than in idiopathic autism and control groups ($p < 0.0001$).

Figure 5. In the oldest subject examined postmortem in the dup(15) group (39-year-old autistic female), and two subjects with autistic disorder of unknown etiology (51 and 52 years old), numerous diffuse $A\beta_{1\text{-}40/42}$-positive plaques were detected in the cortical ribbon (a, b, c, respectively).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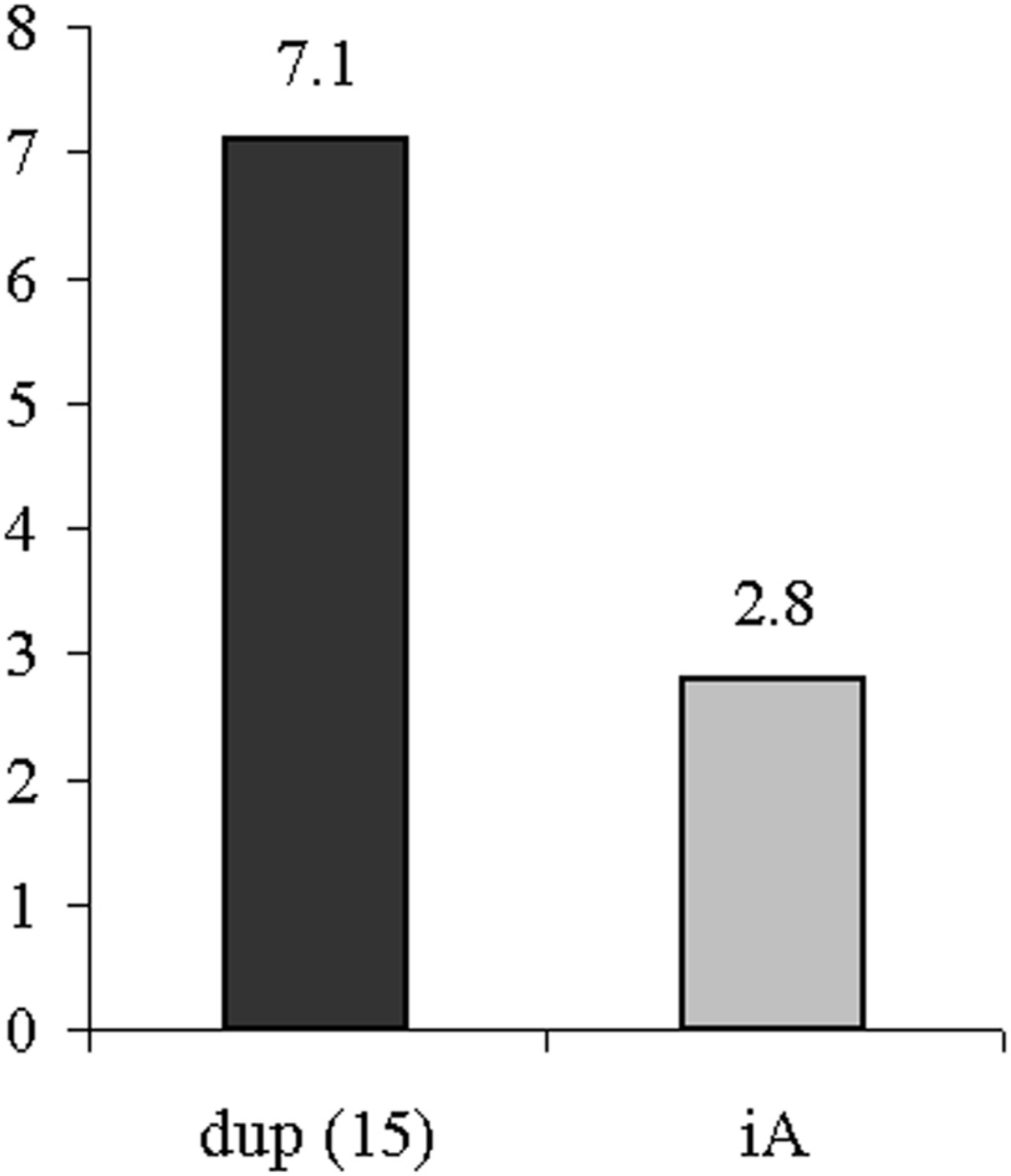
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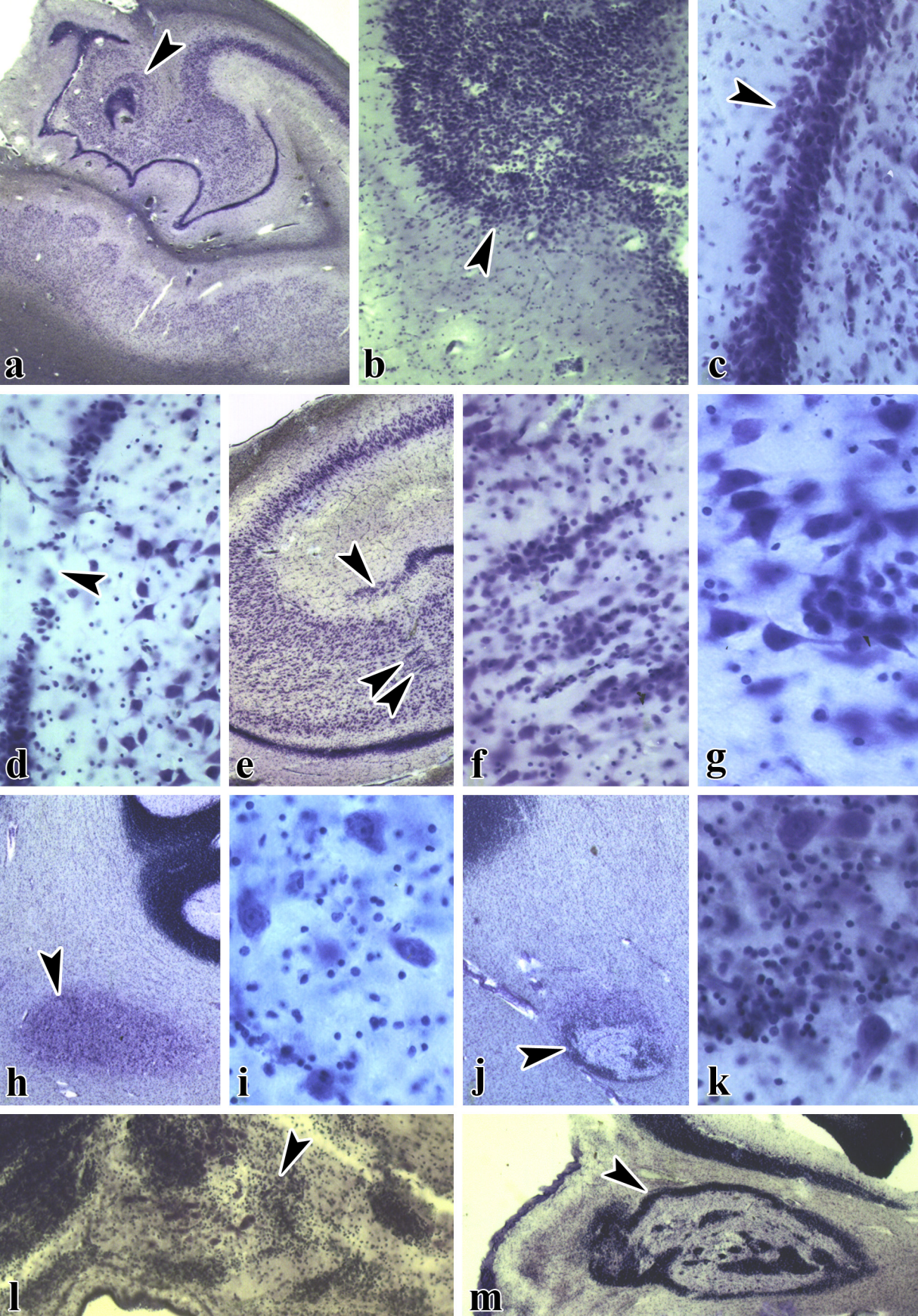
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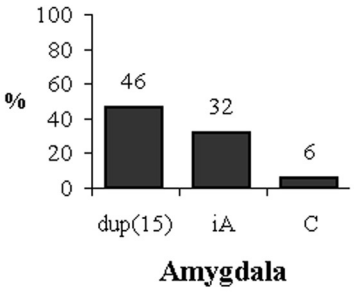
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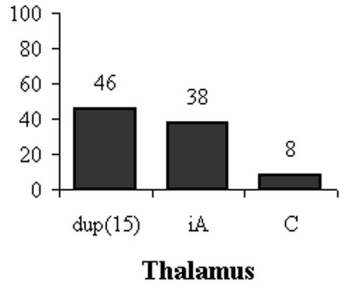
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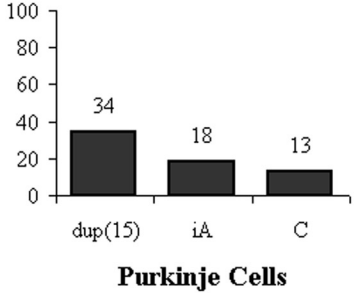
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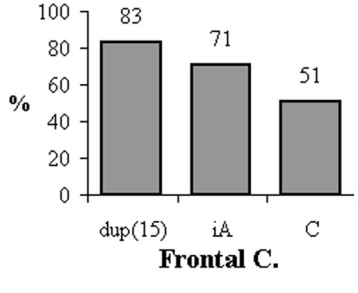
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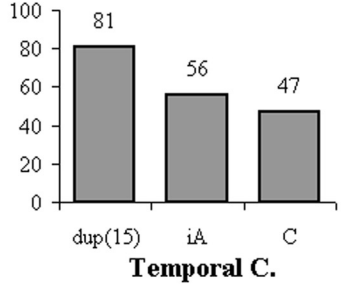
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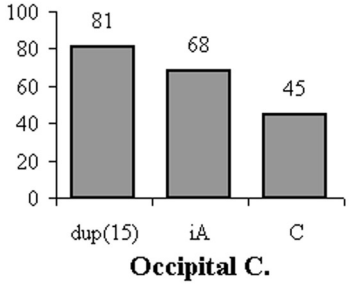
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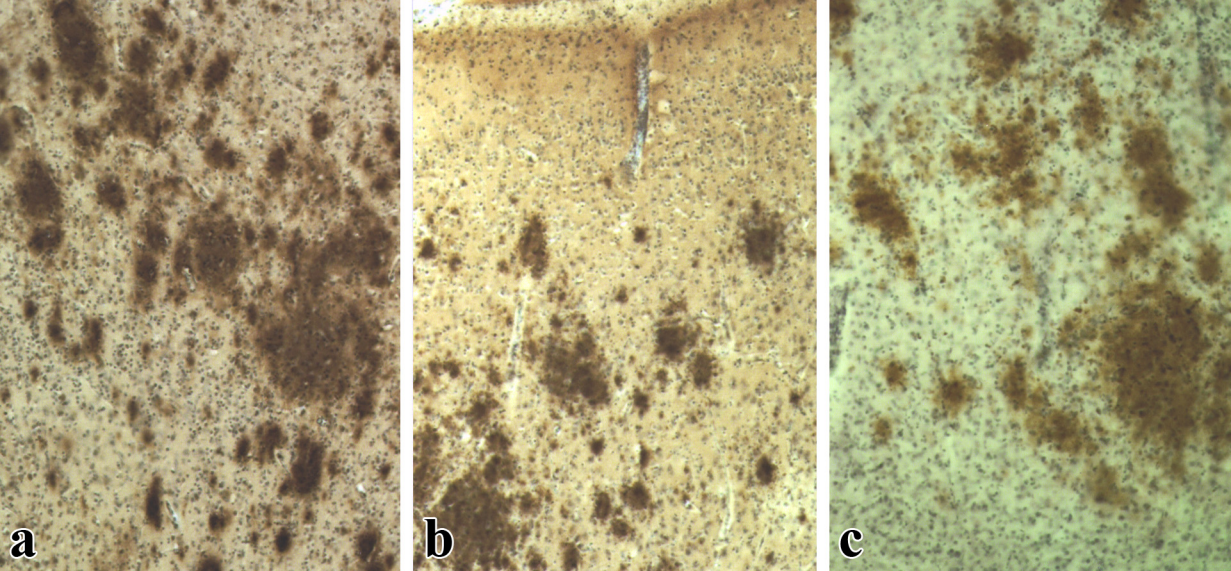
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Brain region– and neuron-type–specific delay of neuronal growth desynchronizes brain development and maturation in autism

Abbreviated Article Title: Desynchronized brain development in autism

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Abstract The study expands corticocentric theory with evidence that autism is associated with delayed and desynchronized neuron growth in early childhood in both cortical and subcortical gray matter and accelerated but still desynchronized neuron growth in late childhood and adulthood. The volume of the neuronal soma and the nucleus was estimated in 16 brain structures and their 19 cytoarchitectonic subdivisions in 13 autistic and 14 control subjects with ages ranging from 4 to 64 years. A significant deficit of neuron soma volume ($p < 0.001$) was detected in 89% of the structures examined, including all 16 brain structures and 15 of 19 of their anatomical subdivisions in 4-8 year old children with autism. A very severe volume deficit in 17%, severe in 44%, moderate in 22% and mild in 17% of brain structures of autistic subjects is a sign of desynchronized development of anatomically and functionally related neurons that may explain social and communication deficits, and restricted repetitive and stereotyped patterns of behavior. Reduction of the developmental deficit from on average 19.6% in 4-8 year old to 8.8% in >8 year old subjects, indicates delayed acceleration of growth of neurons in late childhood and adulthood. Brain region and neuron-type specific volume deficits reflect desynchronized neuron and neuronal networks growth. The most severe delay in 4-8 year old autistic children suggests that deregulation of brain development before the 4th year defines autism encephalopathy and dysfunction for life.

Introduction

Autism is characterized by disrupted development of social and communication skills; restricted, repetitive and stereotypical patterns of behavior, interests and activities; and onset prior to 3 years of age [77]. Nearly 50 % of subjects later diagnosed with autism have the onset of functional alterations between 14 and 24 months, with all three diagnostic functional domains affected by age 24 months. Behavioral worsening in the second year paralleled by slowing of development [72] coincides with the first global marker of abnormal brain development, a rapid increase in head circumference at the age of 1 to 2 years [27, 31, 33, 35, 54]. Normal head size at birth but larger than normal head size in 90 % of 2- and 3-year-old children with autism has been reported [26]. However, a slower rate of brain growth between 2and 4 years of age [19,26,54,108] results in only a 2 % brain overgrowth in adults [94] or even a smaller brain size in comparison to control subjects [51, 71]. The link between the deviation from the normal trajectory of brain growth and the severity of disease suggests a contribution of these changes to the clinical phenotype [27]. However, microcephaly in 15 % of autistic subjects indicates that brain overgrowth is not a sine qua non developmental abnormality in autism [41]. Kemper and Bauman [66] reported higher than expected brain weight in eight of 11 children with autism younger than 12 years of age, but lower brain weight in six of eight individuals with autism older than 18 years of age. Smaller brain weight by 300 g (1,177 g) in subjects diagnosed with dup(15) autism compared to idiopathic autism (1,477 g) suggests that the etiology of autism may be a major determinant of brain size [119].

The second global marker of abnormal brain development is a different rate of growth of various brain regions. Overgrowth of the frontal and temporal lobes and the amygdala but not occipital lobe parallels brain overgrowth in 2- to 4-year-old autistic children. Accelerated growth in the brain regions involved in cognitive, social and emotional functions, and language development suggests that these structural alterations contribute to functional deficits [19, 20, 26, 54, 108].

The third developmental pathology observed are the brain region–specific alterations in the number of neurons. Neocortical studies have revealed a 67 % increase in the number of neurons in the prefrontal cortex [30] and a 53 % increase in the ratio between von Economo neurons and pyramidal neurons in the fronto-insular cortex [100]. However, a reduced number of neurons has been reported in the fusiform gyrus in autism [114]. Qualitative [65, 123] and quantitative studies [8, 40, 73, 98] revealed a regional decrease of the number of Purkinje cells and prenatal loss of Purkinje cells [120, 121].

Reduced neuron size is the fourth developmental defect reported in the neuropathological studies of Bauman and Kemper [14]. The authors demonstrated reduced neuronal size and increased cell-packing density, which are considered markers of an immature brain [42]. Casanova et al [21,22] reported reduced size of neurons and their nuclei in the cortex of autistic subjects and proposed that these alterations impair connectivity between cortical regions and contribute to the autistic phenotype. The reduced perikaryon volume of pyramidal neurons in the inferior frontal cortex in Brodmann areas 44 and 45 of autistic subjects was considered a sign of impairment of neuronal networks relevant to communication and social behaviors [61], whereas the reduced volume of neurons in layers V and VI in the fusiform gyrus was linked to abnormalities in face perception [114].

The fifth type of neuropathological change are qualitative developmental defects of (a) neurogenesis with focal subependymal nodular dysplasia, (b) neuronal migration with the appearance of hippocampal, cerebellar,

periventricular and white matter heterotopias, and (c) multifocal dysplasia in the neocortex, archicortex, hippocampus and cerebellum [118]. A different spatial distribution and a 2.5-fold increase in the prevalence of these developmental abnormalities in dup(15)/autism suggests that the etiology of autism determines the topography and prevalence of brain developmental abnormalities and clinical expression, including prevalence, age of onset and severity of seizures [119].

The prevalence of research focused on the cortex results in a mainly corticocentric theory of autism [44, 47]. However, all three diagnostic modalities of autism engage subcortical structures including (a) the amygdala, in processing social information and involved in emotional interpretation, fear and anxiety [2, 11, 124]; (b) the thalamus, involved in language functions, attention, anxiety and obsessive thinking [-85-87]; (c) the striatum, linked to repetitive motor behaviors, compulsions and rituals [32, 99, 106]; and (d) the brainstem and cerebellar deep nuclei, integrating a cerebellar role in motor functions, language and cognition, and eye motion control [75, 102]. Cholinergic neurons in the nucleus basalis of Meynert control the cortical mantle and play a modulatory role in anxiety, arousal and emotional and motor responses [69. 84], whereas the substantia nigra's dopaminergic neurons modulate striatal functions including repetitive behaviors.

The aim of this study was to test the hypothesis that subcortical structures are affected by developmental alterations and contribute in parallel with cortical networks to global brain developmental defects of connectivity and resulting functional deficits in autism. To test this hypothesis of delayed and desynchronized growth of neurons in early childhood, the neuron volume was compared in autistic and control subjects from 4 to 8 years of age in 16 brain subcortical structures, the cerebellum and the archicortex. Nineteen subregions (layers, sectors, nuclei) were examined to detect signs of desynchronized neuronal growth within individual anatomical brain subdivisions. To test the hypothesis that developmental defects of early childhood are partially corrected in late childhood and adulthood, the volume of neurons in 4- to 8-year-old individuals with autism was compared with that of 9- to 64-year-old subjects with autism.

Material and methods

Clinical inclusion and neuropathological exclusion criteria

Morphometric methods were applied to 13 brains of autistic individuals from 4 to 60 years of age, and 14 control subjects from 4 to 64 years of age. In the examined autistic cohort, there were 2.2 more males than females (nine males and four females). The proportion between males and females (1.8) was comparable in the control group (nine males and five females).

In the first phase of this study, 38 brains were selected, including 20 from autistic subjects and 18 from controls. To reduce the risk of distorted results from the morphometric studies, clinical inclusion criteria and neuropathological exclusion criteria were used. Two cases did not meet the Autism Diagnostic Interview-Revised (ADI-R) criteria for a diagnosis of autism. Five autistic brains were excluded due to postmortem autolysis (one brain), global hypoxic encephalopathy (three brains) and multiple microinfarcts (one brain). In the control group,

four brains were excluded due to severe autolysis distorting neuronal shapes and sizes. This resulted in a reduction of the autistic group by seven cases (35 %) and the control group by four cases (22 %).

To confirm the diagnosis of autism, the ADI-R [77] was administered retrospectively (Table 1). The intellectual disability of eight subjects (61 %), evaluated with the Wechsler Intelligence Scale for Children III and the Woodcock-Johnson Tests of Achievement-Revised, was in the range of mild to severe. Seizures were reported in seven of the 13 subjects (54 %). In five cases, death was seizure-related (38 %). Self-injurious behavior was reported in six cases (46 %), aggression in four (31 %), hyperactivity in three (23 %), obsessive compulsive disorder in two (12 %) and depression and mania in a single case for each.

Tissue preservation and morphometric methods

The cause of death in the cohorts was characterized by the neuropathological report [118]. Postmortem interval (PMI), corresponding to the period between death and autopsy, ranged from 6 to 28 h in the control group (16.7 h on average; SD 7 h) and from 8 to 50 h in the autistic group (21.5 h on average; SD 12 h). The difference in the PMI between these two cohorts was not significant (Table 2). The structures examined were 10 right and two left hemispheres in the autistic group, and 12 right and two left hemispheres in the control group.

The average weight of the autistic subjects' brains (1,439 g) was not significantly different than that of the control brains (1,372 g). The brain hemisphere with cerebellum and brainstem was fixed with 10% buffered formalin for on average of 408 days in the control group (from 52 to 1819 days; SD 490 days). The average time of fixation in the autistic group was 848 days. The brains were dehydrated in a series of ascending concentrations of ethyl alcohol. The average time of dehydration was 36 and 38 days in the control group and autistic group, respectively. Dehydration was associated with a reduction of brain hemisphere weight by 47 % (SD 7 %) on average in the autistic group and by 45 % (SD 7 %) in the control group. The difference between loss of brain weight during dehydration in the autistic and control groups was not significant. Brain hemispheres were embedded in 8% celloidin [56]. Serial 200-μm-thick sections were stained with cresyl violet and mounted with Acrytol.

The neuropathological examination was employed (a) to identify the type, distribution and severity of qualitative developmental changes and (b) to eliminate brain samples affected by pathology not related to autism, changes associated with mechanisms of death or postmortem autolytic tissue degradation. On average, 120 cresyl violet–stained serial hemispheric sections were examined per case in a blinded fashion with regard to diagnosis. The results of the study of the developmental abnormalities in these cohorts were previously summarized [118]. In summary, the neuropathological evaluation revealed a broad spectrum of focal developmental alterations in the 13 examined autistic brains, including (a) subependymal nodular dysplasia, (b) subcortical and periventricular heterotopias and (c) neocortex, archicortex, dentate gyrus, cornu Ammonis and cerebellar dysplasia. The pathology detected in 92 % of the autistic brains reflects focal modifications of neurogenesis, migration and alterations of the cytoarchitecture. The absence of these changes in the examined structures in the control brains indicates that the detected alterations are autism-associated.

The morphometric measurements were performed without knowledge of the subject's age, severity of mental retardation, gender, clinical diagnosis or neuropathological status for the material being analyzed. The

volume of neurons and their nuclei were estimated in 16 brain structures or neuronal populations including those contributing to mind, emotions, social interactions, memory (amygdala, entorhinal cortex, cornu Ammonis and claustrum); the striatum motor system, which controls ritualistic movements (caudate n., putamen, globus pallidus) and the reward system, which probably enhances repetitive behaviors (n. accumbens); the thalamus and two parts (magnocellular and parvocellular) of the lateral geniculate body (LGB); the dopaminergic system (substantia nigra) and the acetylcholinergic system (the nucleus basalis of Meynert; NBM); Purkinje cells, dentate nucleus in the cerebellum and the inferior olive in the brainstem (Fig. 1). Moreover, 19 cytoarchitectonic subdivisions (layers, nuclei, sectors) of these structures were also examined.

Neuronal morphometry was performed at a workstation consisting of an Axiophot II (Carl Zeiss) light microscope with Plan Apo objectives 1.25x (numerical aperture, N.A., 0.15); 2.5x (0.075), and 40x (N.A. 0.75), a specimen stage with a three-axis computer-controlled stepping motor system (Ludl Electronics; Hawthorne, NY, USA), CCD color video camera (CX9000 MBF Bioscience) and stereology software (Stereo Investigator and Nucleator, MicroBrightfield Bioscience, Inc., Williston, VT, USA). The volume of neurons and neuronal nuclei was estimated with the nucleator method [50] using MicroBrightfield software (Nucleator). The parameters and procedures that were applied to estimate the volume of the neuronal soma and neuronal nucleus in 35 brain structures and their subdivisions are presented in Table 3. To eliminate bias related to sectioning defects, 5-µm top and bottom guard zones of the dissector were applied. To preserve the same standard of evaluation of neurons of different size and shape, five rays were used in cellular measurements in all 35 regions of interest. Grid size and the virtual counting space were designed for each brain structure individually to adjust to the size and shape of the region of interest, and to reduce the standard deviation (SD) and the coefficient of error (CE, Schaffer). The number of virtual counting spaces ranged from 34, in very small subdivisions, such as CA4 sector (CE, 0.003), to 664 for Purkinje cells with significant differences in the cell size, shape and orientation (CE, 0.002).

Tissue and medical records were handled in accordance with the NIH Guide for use of human tissue. The research project and protocols were approved by the Institutional Review Board of the New York State Institute for Basic Research in Developmental Disabilities (IBR). The tissue was obtained from the Harvard Brain Tissue Resource Center, the Brain and Tissue Bank for Developmental Disorders of the National Institute of Child Health and Human Development at the University of Maryland, the Brain Bank at IBR, Staten Island, NY, and the Mount Sinai Medical School, Bronx, NY. Tissue samples were coded with the number assigned by the brain banks, and this ID was the only identifier of the tissue, MRI scans, and clinical records. The Autism Tissue Program (Autism Speaks, Princeton, NJ) provided access to brain tissue samples and to the database (www.atpportal.org) with coded and anonymous detailed characteristics of the autistic subjects, including the subject's age at time of death, clinical diagnosis, cause of death, PMI and fixation time.

Statistical analysis

This study determined the effect of autism on neuronal soma and nucleus volume, with age-matched normal controls as a comparison group. Principal analyses treated individual neurons as the unit of analysis, with autism as a fixed factor. The study design is a clustered sampling of neurons, with individual cases representing clusters. This method

takes into account the precision achieved by the measurement of thousands of neurons while accounting for the effects on the sample variances of sampling a limited number of cases. Data have been post-stratified to adjust for the differing numbers of neurons sampled per individual so as to weight each individual equally. Analyses were carried out separately for cases from 4 to 8 years of age at death and for those older than 8 years of age at death, with controls selected to provide age-matching on the group level. Any data points lying more than 1.5 times the interquartile range below the 25th percentile or an equal amount above the 75th percentile for each structure or subdivision were considered outliers and omitted from analyses. Removed defective records accounted for 0.3% of all records. As comparisons were carried out across 16 regions and 19 cytoarchitectonic subregions, *p* values of 0.01 or less were considered statistically significant.

Preliminary analyses regressed neuron size across all structures, normalized by expressing each neuron's size as a proportion of the size of the mean control neuron size for that structure, on PMI (in hours), fixation time (in days), brain weight (in grams), brain weight loss during processing and dehydration (as a percentage), duration of dehydration (in days), autism status and log age (in years). This analysis resulted in detection of significant effects of each of these factors and potential confounders of autism's effects on each of these factors. Potential confounders of autism's effect on neuronal size accounted collectively for 1.09% of the variance in size. Autism status, when entered into the model, raised the explained variance to 1.91 %, an increase of 0.82 percentage points. As autism status univariately explained 1.45 % of variance, all potential confounders considered together diminished the variance explained by autistic status by approximately 43 %, while autism remained by a large margin the strongest predictor in the model. The analyses were conducted using version 11.1 of the Stata statistical package (Stata Corp 2011).

Results

Developmental deficit of neuronal soma and nucleus volume in autistic subjects

Sixteen brain regions were selected to detect potential developmental abnormalities in brain structures contributing to the autism phenotype. The examined regions revealed a broad spectrum of brain structure– and neuron type–specific differences. In the 4- to 8-year-old control subjects, the range of volumes of the examined neurons extended from very large neurons such as Purkinje cells (11,643 μm^3), dopaminergic neurons in the substantia nigra (9,008 μm^3) and cholinergic neurons in the NBM (8,372 μm^3) to much smaller neurons in the striatum, including the putamen (1,316 μm^3), caudate nucleus (1,194 μm^3) and nucleus accumbens (1,174 μm^3) (Fig. 2) (Supplemental Table 1). The lower volume of neuronal soma strongly correlated with an increase in the percent of cell volume occupied by the nucleus (r = 0.86) (Fig. 3). The proportion of the cell body occupied by the nucleus ranged from only 5 % in such large neurons as Purkinje cells to 24 % in the smallest neurons in the nucleus accumbens.

The application of the nucleator to four autistic subjects and to four age-matched control subjects 4 to 8 year of age revealed a significantly reduced volume of neurons in all 16 brain regions ($p < 0.001$) in the autistic subjects. However, the reduced volume of the neuronal soma varied over a very broad range from 4 % to 34 %. An arbitrary categorization of the severity of developmental deficit identified two brain regions affected with a very

severe (> 30 %) neuron volume deficit, seven regions with a severe (20 %–30 %) deficit, four regions with a moderate (10 %–20 %) deficit and three regions with a mild (< 10 %) volume deficit (Fig. 4; Supplemental Table 1). A very severe volume deficit was found for the nucleus accumbens (-34 %) and its Purkinje cells (-31 %). A severe volume deficit was detected for the claustrum (-29 %), thalamus (-27 %), globus pallidus (-27 %), dentate nucleus (-25 %), entorhinal cortex (-23 %), amygdala (-22 %) and nucleus basalis of Meynert (-22 %). A moderate volume deficit was detected in the putamen (-17 %), caudate nucleus (-16 %), inferior olive (-14 %) and magnocellular lateral geniculate body (LGB) (-12 %). A mild, but still statistically significant deficit was found for the substantia nigra (-5 %), parvocellular **l**ateral geniculate nucleus (LGN) (-5 %) and cornu Ammonis (-4 %).

The average neuronal soma volume deficit for 16 brain structures in the 4- to 8-year-old children with autism was 19.6 %, but in the cohort of subjects with autism older than 8 years of age and less than 64 years of age, the deficit was reduced to an average of 8.8 %. In three structures (thalamus, putamen, magnocellular LGB), the volume of neurons was not significantly different from that in controls. In this older cohort, the volume of neurons in the inferior olive and substantia nigra exceeded control levels by 9 % and 5 %, respectively ($p < 0.001$).

The mean volume of the neuronal nucleus was significantly less in all 16 structures examined in the cohort of autistic 4- to 8-year-old subjects than in controls (Fig. 5; Supplemental Table 2). The nuclear size deficit was more pronounced than the neuronal soma deficit. Specifically, a 40–42 % deficit was found for four regions (nucleus accumbens, thalamus, claustrum and dentate nucleus). However, the increase of the nuclear volume in the older than 8-year-old autistic cohort was more prominent than the increase of the neuronal soma volume. In this age cohort, the nuclear volume deficit was found in only six regions, including the claustrum, amygdala and caudate nucleus with more than a 10 % deficit. In four regions, nuclear volume equaled the control levels, and in six regions, nuclear volume in the autistic cohort exceeded the control level ($p < 0.001$).

In control subjects, the volume of the neuronal perikaryon correlated with the nuclear volume ($r = 0.60$ and $r = 0.65$, respectively). A similar correlation was detected in the 4- to 8-year-old autistic subjects ($r = 0.65$) and > 8–year-old autistic subjects ($r = 0.78$). The observed correlations in both age groups for the autistic and control subjects indicate that the different rate of neuronal growth in the 16 examined neuronal populations did not change the neuronal type–specific ratio between the cell soma and nucleus volume.

Different rate of neuronal volume deficit in 19 cytoarchitectonic subdivisions

Estimation of the developmental volume deficit also revealed significant differences between neurons within 19 cytoarchitectonic subdivisions, including the four sub-nuclei of the amygdala, four sectors of the cornu Ammonis, four layers in the entorhinal cortex, four nuclei in the NBM and three major parts of the substantia nigra (Fig. 6; Supplement Table 3a,b).

The amygdala with a dense network of local connections between individual nuclei revealed signs of severe volume deficit within all four nuclei, including the accessory basal (20 %), lateral (25 %), central (26 %) and basal (30 %) nucleus. All differences between the autistic and control 4- to 8-year-old subjects were significant ($p < 0.001$). The deficit in the cohort of autistic subjects older than 8 year of age was moderate and uniform (in a range of 10–16 %). The difference in developmental deficit was estimated for six inter-nuclear comparisons per age group.

The lack of significant difference in only one comparison in the younger group and three of six in the older group suggests a partial (50%) synchronization of the rate of neuronal growth in the amygdala subdivisions in the autistic subjects older than 8 year of age.

In the cornu Ammonis sectors CA1-CA4, which are integrated by numerous connections between individual sectors, the developmental neuron volume deficit was moderate (-16 %, -18 %, -12 %, -17 %, respectively). A comparison of the rate of deficit between the sectors revealed that only two of six comparisons in the younger, and one of six in the older cohort revealed no statistical difference.

Evaluation of the four layers of the entorhinal cortex revealed severe developmental deficits in tlayers III, V and VI (by 23 %, 29 %, and 21 %, respectively), but the deficit in the stellate neurons in the islands of the second layer was only moderate (11 %) (Supplement, Table 3a). Comparison of the range of the developmental deficits between the four examined layers demonstrated a comparable developmental trajectory in one of six comparisons in the 4- to 8- year-olds and three of six comparisons in the autistic subjects older than 8 years old, s suggesting an approximately 50 % correction of the desynchronized neuronal development in the older cohort in the archicortex mediating connections between the neocortical association areas and the hippocampal memory system.

Striking developmental differences were observed within the two major sources of brain neurotransmitters, with a mild (5 %; $p < 0.000$) developmental deficit in the substantia nigra but a severe (22 %, $p < 0.000$) developmental deficit within the cholinergic nucleus basalis of Meynert when compared with the control cohorts (Supplement 3a). The difference ($p < 0.001$) between the global measures of developmental deficits in each of these neurotransmitter systems indicated a different rate of neuronal growth, reflecting a desynchronized growth of neurons in both cohorts (4–8 and > 8 years of age) in the dopaminergic and cholinergic systems.

To verify the hypothesis of a global desynchronization of brain development, the difference between the rate of neuronal growth in the control and autistic subjects was evaluated in the large complex of anatomically and functionally related structures of the nigro-striatal system. The developmental deficit ranged from very severe in the nucleus accumbens, to severe in the globus pallidus, to moderate in the putamen and caudate nucleus. Analysis of the dopaminergic neurons of the substantia nigra that integrate striatal functioning revealed the smallest (5 %) but a significant developmental volume deficit. The volume deficit was significantly different in nine of 10 comparisons between the five subcomponents of the nigrostriatal system of autistic subjects 4 to 8 years of age and in subjects older than 8 year of age ($p < 0.001$). This pattern reflected desynchronized development of the interacting neurons within the five subdivisions of the dopaminergic nigrostriatal system of the autistic children.

No evidence of developmental volume deficit in the lateral part of the substantia nigra, and a mild deficit in the ventral (-8 %; $p < 0.000$) and dorsal part (-3 %; $p < 0.03$) reflected the relatively limited immaturity of the dopaminergic neurons in the 4- to 8-year-old autistic children. No statistical difference in one of three comparisons of neuronal volume deficits in the three substantia nigra subdivisions in the 4- to 8-year-olds, and in two of three comparisons in the older than 8-year-old subjects suggested only partial desynchronization of the development of neurons within the substantia nigra of the autistic subjects.

The cholinergic system's subdivisions in the 4- to 8-year-old autistic children showed severe (Ch3; 23 %; $p < 0.000$), moderate (Ch2, 19 % and Ch4, 17 %; $p < 0.000$) and mild deficit of neuronal volume (Ch1, 7 %; $p < 0.032$) in comparison to the control 4- to 8-year-old subjects. Detailed analysis showed that only one of six

comparisons of the four NBM subdivisions revealed no difference between volume deficits in the 4- to 8-year-old children. However, in the older than 8-year-old autistic children, there was no significant difference in volume deficits (six of six comparisons), suggesting a strong tendency to synchronized growth of neurons in the NBM subdivisions in late childhood and adulthood.

The thalamus with a severe deficit, magnocellular LGN with a moderate, and the parvocellular LGN with a mild deficit reflected different rates of neuron growth (desynchronized growth) within the complex of brain structures united by similar histogenesis in the 4- to 8-year-old autistic children. In the older than 8-year-old children, one of three comparisons revealed no statistical difference, suggesting a partial correction of the developmental differences between the thalamic subdivisions in the older autistic subjects.

Desynchronized development of the functional networks within the cerebellar cortex, cerebellar nuclei and brainstem was documented by a very severe volume deficit in the Purkinje cells (-31 %), a severe deficit of growth of the neurons of the dentate nucleus (25 %) and a moderate deficit in the inferior olive (-14 %) in the 4- to 8-year-old autistic subjects. All comparisons between these three components of the network integrating brainstem and cerebellum revealed significant ($p < 0.001$) differences in 4- to 8-year-old and > 8-year-old cohorts, suggesting lifelong desynchronization of cerebellar connectivity.

The striking developmental volume deficits found in (a) the smallest neuronal populations such as in the nucleus accumbens (1,174 μm^3 in control subjects and 778 μm^3 in autistic subjects; 34 % volume deficit), (b) medium-size neurons in the thalamus (3,683 μm^3 in control and 2,676 μm^3 in autistic subjects; 27 % volume deficit), and (c) the largest neurons, the Purkinje cells (11,643 μm^3 in control and 8,057 μm^3 in autistic subjects; 31 % volume deficit) suggested that cell volume does not determine the range of developmental deficit. The lack of correlation between cell body volume and volume deficits in the 4-to 8-year-old subjects as well as between the nucleus volume and developmental volume deficits (r = 0.049; Pearson correlation) confirmed the hypothesis that the volume of neuron or cell nucleus is not a predictor of developmental deficits of neuronal volume in autistic subjects.

Discussion

This morphometric study of the brains of subjects diagnosed with idiopathic autism is based on ADI-R inclusion criteria and neuropathological exclusion criteria [119] and a research design supporting the project's aims. The applied criteria led to the exclusion of 35 % of brains of autistic subjects and 22 % of brains of control subjects, resulting in a reduction of potential distortions of the stereological estimates by co-morbidities not related to autism, and pre- or postmortem changes. These procedures diminished the size of the autistic cohort to 13 cases and the control cohort to 14 cases. However, the preservation of four pairs of brains of autistic and control subjects 4 to 8 years of age, and of brains of nine autistic and 10 control subjects older than 8 years of age allowed detection of the differences between patterns of developmental changes in early childhood and neuronal maturation in late childhood/adulthood. The examination of 16 regions and 19 subregions (35 structures and neuronal populations) in the same brains of autistic and control subjects by four neuropathologists blind to demographics and using the same equipment, software and serial hemispheric sections resulted in an increase in consistency and a reduction of

distortion by subjective evaluation and by such technical factors as tissue shrinkage during processing, staining and handling.

The properties of the examined postmortem cohort, male/female ratio, range of intellectual deficit, prevalence of epilepsy and sudden unexpected death in epilepsy (SUDEP) are so close to those reported in large demographic and clinical studies of autism, that the detected temporal and spatial patterns of developmental brain alterations can be considered as representative of idiopathic autism. The 2008 Centers for Disease Control and Prevention (CDC) study of 8.4 % of the US population of 8-year-old children reported a 1 in 88 overall prevalence of autism spectrum disorder (ASD) with a 1 in 54 prevalence for males and a 1 in 252 prevalence for females ($p < 0.01$) with a male to female ratio of 4.7 to 1. However, differences between sex ratio ranged in the 14 participating sites from a low of 2.7 to 1 in Utah to a high of 7.2 to 1 in Alabama [113]. The male to female ratio of 2.2:1 in this postmortem-examined cohort was closest to that found in Utah. The presence of eight (61 %) subjects with signs of cognitive impairment ranging from mild to moderate to severe in this postmortem-examined cohort reflects the approximate prevalence of cognitive impairment reported in large U.S. cohorts. In the CDC review, 38 % of children with ASD were classified as having intellectual disability with an IQ < 70, and 24 % were in the borderline range with an IQ of 71–85 [113]. The general rate of death among autistic subjects is reportedly 5.6 times higher than expected [49]. Epilepsy and cognitive impairment–related accidents contribute to most of the deaths [9, 49, 107] reported as SUDEP that occurs in patients with known epilepsy [37, 74]. Approximately 33 % of individuals with autism are diagnosed with epilepsy [112]. The higher prevalence of epilepsy (54 %) coincides with a high rate of SUDEP (38 %) and reflects the difference between the prevalence of epilepsy in clinic-based samples and in this postmortem-examined cohort.

Cortico-centric theory of autism

Recent genetic findings along with anatomical and functional imaging studies suggest an ASD model in which higher-order association areas of the brain that normally connect to the frontal lobe are partially disconnected during development (“a developmental disconnection syndrome”) [29, 44, 47]. However, the disconnection in ASDs is not primarily a disruption of previously connected regions, but rather a failure of them to develop normally [47]. Functional MRI and postmortem studies of autism providing evidence of cortical developmental abnormalities have become the foundation for several theories of autism, including a theory of a deficit of long-range connectivity, an increase in local short-range connectivity, disrupted cortical synchronization [17, 28, 44, 60, 95] and a defective theory of mind, or mind blindness [10, 43, 45, 70]. The hypothesis of a deficit of long-range connectivity is based on functional imaging showing abnormal activity patterns between distant but functionally and anatomically related cortical regions [3, 23, 24, 34, 63, 64, 67]. An increase in local short-range connections is supported by the observation of an increase of the white matter volume [57, 58], enhanced activity focused on tasks requiring local processing of information [52] and reduced selectivity [16]. Disrupted cortical synchronization appears to be a feature of autism evident between the ages of 1 and 3.5 years. The strength of synchronization correlates positively with verbal ability and negatively with autism severity [34]. A global and regional reduction of the corpus callosum size is considered to be a structural indicator of deficient connectivity between the hemispheres [115]. Postmortem

studies have confirmed cortical alterations including decreased neuronal size in Brodmann areas 44 and 45 in the inferior frontal cortex, which is involved in language processing, imitative functioning and social processing networks [61], and in the fusiform gyrus, which is involved in the face-processing system [114], as well as in the amygdala [104]. Bauman and Kemper's [15] neuropathological studies expanded the list of brain structures that show reduced neuronal sizes to the limbic system including the hippocampus, amygdala and entorhinal cortex, as well as the mamillary body, medial septal nucleus and anterior cingulate.

Delay and desynchronization of subcortical structure development as an indicator of global encephalopathy in autism

In contrast to the majority of studies focused on the link between neocortical abnormalities and the diagnostic features of autism, this study was concentrated mainly on subcortical structures and the archicortex. Neuronal volume deficits in all the examined cortical and subcortical neuronal populations and brain structures reflect a delay of neuronal growth as a sign of global immaturity of the brain. The severity of developmental delay was brain region– and neuron type–specific and ranged from 4 % in the cornu Ammonis to 34 % in the nucleus accumbens. The dynamic nature of the developmental alterations was observed as partial or, in a few brain structures, total correction of developmental delays. However, it is not known whether autistic children are born with immature neurons, or whether the growth and maturation of neurons slows down after birth, or whether maturation slows down at approximately 1.5 years of age, when regression is commonly observed in autistic children, as reported by Ozonoff et al. [88]. The detected desynchronized development of neurons and neuronal networks could contribute to a loss of the structural and functional integrity of cortical and subcortical structures. The broad spectrum of functional links of the examined brain structures to functional deficits and behavioral alterations considered the diagnostic markers of autism suggests that neuronal growth delay and immaturity is a major contributor to the clinical autism phenotype.

The spatial pattern of delayed and desynchronized development of neurons in the brains of autistic subjects corresponds not only to the three diagnostic domains of autism but also to the broader autistic clinical phenotype. It may also reflect changes contributing to the intellectual deficits seen in autism, where 62 % were reported to have an IQ < 85 % (U.S. Department of Health and Human Services 2012). Although autism and intellectual deficits often overlap, autism is not synonymous with global intellectual disability or mental retardation [47]. Common findings are seizures in 33 % [112]; impulsivity, temper tantrums, self-injurious behavior and aggression in 20–71 % [5, 7, 13, 36, 62]; anxiety in 84 % [83]; depression in 50 % [48]; sensorimotor deficits and impairments of gross and fine motor function, including motor stereotypes, hypotonia, limbic apraxia [97]; abnormal or paradoxical responses to sensory stimuli in 42 % to 88 % [68] and abnormal sleep pattern in 65 % [81, 110]. A number of studies have reported that individuals with autism and more severe intellectual deficits have higher levels of self-injurious behavior [13, 18, 25, 91], tantrums, aggression and destruction of property [4, 78]. The progress in neuropathological studies will likely result in a further stratification of the spatial and temporal patterns of developmental neuropathology corresponding to both the core autism clinical diagnostic phenotype, and the pathology contributing to other autism-associated clinical alterations. Genetic differentiation of social impairment

from communication deficits and stereotypical repetitive behaviors suggests that different brain regions and neuronal networks contribute to the different features of autism [53]. Voineagu et al [117] found that whereas 174 genes were differentially expressed between control Brodmann areas 9 and 41, none of these genes were differentially expressed in the same regional comparisons among the ASD cases. One may speculate that developmental delays in subcortical structures are also associated with the altered gene expression specific for affected neuronal populations and brain subdivisions and correlate with the broader spectrum of functional deficits observed in autism.

Clinicopathological correlations

All subjects with autism display features of a deficit of social behavior, including abnormalities in social reciprocity and difficulties in the use of eye contact, facial expression and social motivation [77, 93]. Neurobiological models of social cognition suggest that in the neuronal network engaged in social cognition, including the amygdala, the cortex of the temporal superior sulcus and the fusiform gyrus, it is the amygdala that is responsible for labeling with emotional meaning [11, 124]. Moreover, the amygdala plays a role in the detection of threats and mobilizing an appropriate behavioral response including fear and anxiety [2], and anxiety is observed in 84 % of autistic children [83]. Neuropathological data [65] as well as results of structural [103, 108] and functional neuroimaging [12] provide evidence that the amygdala is affected in autism and that pathology of the amygdala may contribute to the clinical deficits of autistic subjects. The 22 % reduction in the volume of neurons ($p < 0.001$) in the amygdala of the 4- to 8-year-old autistic subjects suggests that during the most critical stage of development of social behaviors and emotional relationships, the growth of neurons in the amygdala is delayed. The reduced difference in neuronal volume to 12 % ($p < 0.001$) in older children and adults, and the partial improvement of communication and social behavior progressing with age [79, 90, 92] suggest that a partial normalization of neuron growth may contribute to a clinical improvement in functional domains controlled by the amygdale, including social interactions.

Smaller and more densely packed neurons were found in various portions of the hippocampal formation, entorhinal cortex and medial nuclei of the amygdala [14, 65]. The finding of reduced size of neurons of a comparable range in the entorhinal cortex (-23 %; $p < 0.001$) and in the amygdala (-22 %; $p < 0.001$) in the 4- to 8-year-old autistic children, but only a 4 % neuronal volume deficit in the cornu Ammonis (-18 %; $p < 0.009$), suggests that the limbic system is developmentally delayed, but the range of delay of closely connected and interacting structures is significantly different. The very small delay of neuron growth in the cornu Ammonis, which is involved in the storage of information provided by the entorhinal cortex and amygdala, with a five times more severe delay of neuronal growth in the entorhinal cortex and the amygdala, illustrates desynchronized limbic system development. The observed pattern of changes may correspond to both restricted interests and abilities, and a high efficiency in some activities requiring memory.

The claustrum receives inputs from many cortical areas, integrates multiple inputs into a new signal and redirects sensory information throughout the striatum and thalamus. Interconnectivity with subcortical nuclei and sensory cortical areas indicates the claustrum's involvement in sensorimotor integration and potentially the most complex human brain function —consciousness, as well as in higher orders of functionality enabling the organism to

rapidly adapt to the subtleties and nuances of a changing environment [39]. The attraction to routines and sameness appears to be one of the very striking behavioral alterations characteristic of autism. It appears that claustrum immaturity, reflected in the neuronal soma deficit of 29 % in children and of 17 % in adults, and the very striking deficit of neuronal nucleus volume (42 % and 22 %, respectively), may be responsible for the claustrum neurons' functional impairment and deficits of adaptability and consciousness.

The striking differences in the range of developmental delay of the claustrum neurons, and the neurons receiving claustrum projections and projecting to the claustrum, suggest that claustrum dysfunction is associated with or caused by a desynchronized development of the subcomponents of these multifunctional networks. The claustrum is involved in long-term response potentiation within the claustral–entorhinal–hippocampal system [122], with neuronal volume deficits of 29 %, 23 % and 4 %, respectively. The frontal, temporal, parietal and occipital cortex project to the claustrum [38, 76], whereas the dorsocaudal claustrum (visual claustrum) projects to the visual cortex. Only a slight reduction of neuronal volume in Brodmann area 17 in the occipital cortex [22] and a significant reduction in the mean perikaryal volume of the neurons in layers V and VI (by 21.1 % and 13.4 %, respectively) in the fusiform gyrus [114] are indicative of desynchronized development of the claustro-cortical networks in autism. It may affect the role of the claustrum as an integrator of input from the somatosensory, auditory and visual cortices, as well as from the respective diencephalic relays [109]. The 12% and 5%, respectively, deficit in the neuronal volume in the magnocellular and in parvocellular lateral geniculate nucleus, and the 27% deficit in the neuronal volume in the thalamus indicate developmental desynchronization of claustrum-related regions. The rostral portion of the claustrum projects to the caudate nucleus [6], which is also affected by a 16 % deficit in neuronal volume.

The NBM consists of four major nuclei that send cholinergic, GABAergic and glutamatergic axons to the cortical mantle, amygdala and many subcortical structures [80, 101, 116] and may contribute to the clinical phenotype of autism. The cholinergic drive to the forebrain plays a modulatory role in anxiety, arousal and attention, and is essential for learning and memory tasks [69, 84]. The anteromedial part of the NBM (CH4 complex) acts as the cholinergic relay for transmitting limbic and paralimbic information to the neocortex, thereby influencing complex behavior (integrated emotional, and motor responses, learning and memory), according to the prevailing emotional and motivational states encoded by the limbic and paralimbic brain structures. Ch4 neurons respond to the sight and taste of food, visual and auditory information. All the structures that project to the Ch4 are integrative regions of extensive sensory processing or regions of polysensory convergance. The detected delay of NBM neuronal growth by 19 % and their nuclei by 25 % 4 to 8 years of age may reflect the defective function of the cholinergic system in early childhood. The reduced volume of neurons in the NBM of autistic subjects may result in an altered cholinergic innervation of the cortical mantle and contribute to anxiety, arousal, attention deficit and learning difficulties. The outcome of the combination of (a) region/cell type–specific delay of neuronal growth and (b) systemic defects of cholinergic innervation may contribute to the broad spectrum of autistic phenotypes, including communication and social deficits, and repetitive and stereotyped behaviors.

Experimental studies have shown that almost all amygdaloid nuclei project to the thalamus and that their main target is the rostral half of the magnocellular mediodorsal thalamic nucleus [1, 98], which is involved in attention, emotional processing, anxiety, obsessive thinking, agitation and assaultive behavior [85, 86, 87]. The lateral thalamus is closely related to language function, including the mechanical processes for articulation and

respiration. The pallidal input to the thalamus serves to control muscle tone. MRI studies have revealed significantly reduced mean thalamic volume in high-functioning autistic subjects [111]. The very significant delay in the growth of neuronal soma (-27 %; $p < 0.001$) and neuronal nucleus (-42 %; $p < 0.019$) in the thalamus of autistic subjects 4 to 8 years of age indicates that neuronal circuits involved in articulation, attention, anxiety and obsessive thinking belong to the most developmentally delayed brain structures. The normal neuron and cell nucleus in the thalamus in autistic adults suggests a restoration of the morphological features of neurons but without functional normalization.

A reduced number and size of Purkinje cells was noticed in the majority of cerebellar reports in 21 of 29 examined cases [89]. Our study of 4- to 8-year-old children with autism revealed a severe developmental delay of Purkinje cells with significant cell and nucleus volume deficits (-31 %; $p < 0.001$ and -32 %; $p < 0.001$, respectively) and a lesser, but still significant, deficit of neuronal cell body volume (-17 %; $p < 0.001$) in subjects older than 8 years of age. These signs of immaturity coincide with a 40 % decrease in the expression of glutamic acid decarboxylase 67 (GAD67) mRNA in autistic subjects [125] and an increased GABAergic feed-forward inhibition to Purkinje cells by basket cells, suggesting a disruption in the timing of Purkinje cell firing and altered inhibition of the cerebellar nuclei, which could directly affect cerebellocortical output, leading to changes in motor behavior and cognition [126]. The very significant delay of the growth of Purkinje cells and neurons within the dentate nucleus (-25 %; $p < 0.001$) and less so in the inferior olive (-14 %, $p < 0.001$) suggests a developmental heterochronicity with different rates of growth for different cerebellar and brainstem structures.

The autism phenotype includes lower-order repetitive motor behaviors, intense circumscribed patterns of interests and higher-order rituals and compulsions that occur regularly and interfere with daily functioning [46]. An inverse correlation between the caudate nucleus volume and the presence of compulsions and rituals, and difficulty changing routine was detected in 12- to 29-year-old autistic patients [105]. An increased volume of the caudate nuclei was proportional to compulsions and rituals [106]. The volume of the right caudate nucleus correlates with repetitive behaviors in 17- to 55-year-old ASD subjects [59]. Our study revealed that the cellular component of these abnormalities had a reduced volume of neurons in the caudate nucleus and putamen by 16 % and 17 %, respectively, but by 27 % in the globus pallidus and by 34 % in the nucleus accumbens. These findings suggest that repetitive motor behaviors, circumscribed patterns of interests, rituals and compulsions are the functional consequences of the delayed maturation of striatal networks. Because of its anatomy and connectivity, the nucleus accumbens is considered a mixed structure with elements of the striatum and “extended amygdala” [55]. The nucleus accumbens is required for a number of reward-related behaviors, and it processes specific information about reward availability, value and context [32]. The nucleus accumbens plays a crucial role in appetitive behavior regulation [99]. Its projections to motor areas such as the ventral pallidum turn reward information into motivated action [82].

The detected deficits of neuronal growth expand the cortical model of developmental underconnectivity [44, 47, 63, 64] and provide evidence for altered trajectories of development and maturation of the neurons in subcortical structures, the entorhinal and cerebellar cortex, and brainstem. This model implicates a triple effect of subcortical developmental abnormalities, defined as connectivity defects within subcortical structures and between subcortical structures, and defects of connections and interactions between subcortical structures and the cortex.

Neuronal size does not predict the rate of growth delay. It suggests that autism-associated mechanisms that slow down growth of the neuronal body and nucleus are independent of physiological determinants of cell type–specific developmental trajectories. The lack of correlation between the rates of neuronal growth delay within functional networks indicates that the factors integrating the networks do not control the mechanisms of delayed neuronal growth. Similar trends of delayed neuronal growth in the cortex [22, 61, 100, 114] and in subcortical structures support a model of global developmental encephalopathy with neuron type–specific delay of neuronal growth contributing to the core autism phenotype. Significant differences between patterns of developmental alterations in 4- to 8-year-old children and subjects older than 8 years of age suggest that changes in cell structure, function and gene expression occur during both brain development and maturation. One may expect that understanding transcriptome organization may help to identify the molecular markers and mechanisms involved in the delay of subcortical structure development in early childhood and the accelerated growth in late childhood.

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Figures

Fig. 1 Cresyl violet stained three coronal sections from right brain hemisphere (Nr. 295, 343, and 425) and two midsaggitally cut sections from cerebellum (C31 and C61) from a 7-year-old male. They show localization and spatial relationships of 16 examined brain regions including: four striatum subdivisions (caudate nucleus, CN; putamen, Pu; nucleus accumbens, Ac; and globus pallidus, Gp); claustrum, Cl; structures of the limbic system (amygdala, Am; entorhinal cortex, EC, and cornu Ammonis, CA); thalamus, Th; lateral geniculate body (LGB) with magno- and parvocellular parts examined; two neurotransmitter systems with cholinergic nucleus basalis of Meynert, NBM, and dopaminergic substantia nigra, SN. In brainstem, the inferior olive, IO; in cerebellum, Purkinje cells, PC; and neurons in dentate nucleus, DN, were examined. Calibration bar, 20 mm.

Fig. 2 The range of volume of neuronal soma and nucleus in 16 examined brain structures from the smallest neurons in the nucleus accumbens (Ac) to the largest Purkinje cells (Pc).

Fig. 3 The ratio between nucleus and cell body increases from 5 % in the largest neurons (Purkinje cells) to 24 % in the smallest neurons (nucleus accumbens, Ac).

Fig. 4 In 16 examined structures, neuronal soma volume deficit in 4- to 8-year-old (gray bar) autistic subjects ranges from mild (< 10 %), moderate (10–20 %), severe (20–30 %) to very severe (> 30 %). The deficit in > 8-year-old (black bar) autistic subjects is reduced significantly in almost all structures.

Fig. 5 Neuronal nucleus volume deficit is more pronounced than cell body, and developmental correction of nucleus volume reveals trend to increase above the control level in > 8-year-old subjects.

Fig. 6 Differences of the neuronal volume deficit between cytoarchitectonic subdivisions in the amygdala nuclei (accessory basal, AcB; basal, Ba; Central, Ce; and Lateral, La), four layers of the entorhinal cortex (islands of stellate neurons, 1; and layers 3, 5, and 6); four nuclei of the nucleus basalis of Meynert (Ch1, 2, 3, and 4), and three subdivisions of the substantia nigra (dorsal, SNd; ventral, SNv; and lateral, SNl).

Table 1. Autism diagnosis, prevalence of seizures and SUDEP

Case number	Sex	Age (y)	Autism Diagnostic Interview- Revised (ADI-R)				Seizures age of onset and SUDEP
			Social deficits (10)	Communication deficits		Repetitive behavior (3)	
				Verbal (8)	Nonverbal (7)		
1	M	4	14	NA	10	3	—
2	F	5	26	NA	11	4	—
3	M	7	29	NA	14	3	14 months
4	M	8	19	14	NA	4	—
5	F	11	22	14	NA	3	4.5 months SUDEP
6	M	13	28	NA	12	3	2 years SUDEP
7	F	17	15	16	NA	7	—
8	F	21	21	NA	11	7	5 years SUDEP
9	M	22	28	NA	14	6	15 years SUDEP
10	M	23	30	NA	14	8	23 years SUDEP
11	M	36	23	NA	10	6	—
12	M	56	19	8	13	3	—
13	M	60	26	8	14	4	3 years

ADI-R cut-off score shown in a parenthesis.

SUDEP, unexpected and unexplained death that occurs in patients with known epilepsy.

Table 2. Tissue samples, demographics of autistic and control subjects, brain weight and changes during processing

Group	Case number	Sex	Age (years)	PMI (h)	Brain weight (g)	H	Fixation (days)	Dehydration (days)	Weight loss (%)
A	1	M	4	30	1,280	R	4,560	28	49
A	2	F	5	13.2	1,390	R	1,568	28	52
A	3	M	7	25	1,610	R	330	37	55
A	4	M	8	22.2	1,570	R	196	36	45
A	5	F	11	12.9	1,460	L	308	34	52
A	6	M	13	8	1,470	L	75	33	39
A	7	F	17	25	1,580	L	470	36	51
A	8	F	21	50	1,108	R	136	35	43
A	9	M	22	25	1,375	R	1,034	39	38
A	10	M	23	14	1,610	R	533	45	60
A	11	M	36	24	1,480	R	721	37	44
A	12	M	56	3.35	1,570	R	692	38	52
A	13	M	60	26.5	1,210	R	398	39	38
Average				**21.5**	**1,439**		**848**	**36**	**48**
SD				**12.0**	**161**		**1,187**	**5**	**7**
C	1	F	4	17	1,530	R	126	41	49
C	2	F	4	21	1,222	R	233	30	43
C	3	M	7	12	1,240	R	130	41	51
C	4	F	8	20	1,222	R	650	36	51
C	5	M	14	20	1,464	R	1,067	38	44
C	6	F	15	9	1,250	R	372	41	49
C	7	F	20	9	1,340	R	245	37	52
C	8	M	23	6	1,520	R	95	45	41
C	9	M	29	13	1,514	R	89	41	49
C	10	M	32	24	1,364	R	460	37	42
C	11	M	48	24	1,412	L	215	38	39
C	12	M	51	18	1,450	L	1,819	20	26
C	13	M	52	13	1,430	R	158	47	48
C	14	M	64	28	1,250	R	52	37	51
Average				**16.7**	**1,372**		**408**	**38**	**45**
SD				**6.6**	**118**		**491**	**7**	**7**

PMI, postmortem interval; h, hours; H, hemisphere; R, right; L, left; SD, standard deviation.
Fixation: duration (in days) of fixation in 10 % buffered formalin.
Dehydration: duration (in days) of dehydration in ethyl alcohol.
Weight loss: decrease of hemispheric brain sample weight during dehydration in ethyl alcohol.

Table 3. Parameters and procedures applied to estimate the volume of neuronal soma and nucleus

Structures and their cytoarchitectonic subdivisions	**Number of sections examined (per case)**	**Obj.**	**Grid size (µm)**	**Test area frame x height (µm)**	**Mean number of virtual counting spaces (per case)**	**Mean number of neurons measured (per case)**	**CE***
Amygdala	3	40x	1000x1000	80x80x10	356	454	0.01
Thalamus	3	40x	1000x1000	80x80x10	188	313	0.01
Entorhinal cortex LII	6	40x	400x400	60x60x30	109	117	0.003
LIII			800x800	60x60x30	117	139	
LV			600x600	60x60x30	62	122	
LVI			800x800	60x60x30	67	124	
CA1	14	40x	1000x1000	60x60x30	53	139	0.003
CA2			400x400	60x60x30	66	122	
CA3			400x400	60x60x30	79	118	
CA4			600x600	100x100x30	34	130	
Caudate nucleus	4	63x	2000x2000	80x80x30	74	244	0.002
Putamen	4	63x	2000x2000	80x80x30	73	255	
Globus pallidus	4	63x	500x500	180x180x30	117	137	
Nucleus accumbens	3	63x	500x500	180x180x30	134	353	
Magnocellular LGB	4	40x	500x500	100x100x30	114	190	0.002
Parvocellular LGB	4	40x	500x500	100x100x30	123	211	0.002
Claustrum	3	40x	250x250	60x60x10	274	318	0.01
Substantia nigra	9	63x	300x300	80x80x30	108	287	0.002
Nucleus basalis of Meynert	8	63x	300x300	80x80x30	59	153	0.005
Purkinje cells		40x	1800x1800	180x180x30	664	253	0.002
Dentate nucleus		40x	1000x1000	180x180x30	145	253	0.002
Inferior olive		40x	1000x1000	180x180x30	132	260	0.002

*CE, the average predicted coefficient of error of the measured neurons and neuronal nuclei (Schaffer); LGB, lateral geniculate body.

Table S1. The difference between the mean volume of neuronal soma in autistic and control cohorts

Brain structure	Cohorts of 4- to 8-year-old subjects				Cohorts of > 8-year-old subjects			
	Autism	**Control**	***p* <**	**Volume difference (%)**	**Autism**	**Control**	***p* <**	**Volume difference (%)**
	Mean LSE n	Mean LSE n			Mean LSE n	Mean LSE n		
Nucleus accumbens	778 7 1,317	1,174 9 1,227	0.000	-34	1,007 6 2,968	1,031 5 3,496	0.001	-2
Purkinje cells	8,057 97 1,077	11,643 105 1,050	0.000	-31	9,215 63 2,591	11,107 67 2,782	0.000	-17
Claustrum	1,410 13 2,436	1,999 15 2,739	0.000	-29	1,582 13 2,886	1,904 13 3,223	0.000	-17
Thalamus	2,676 35 1,090	3,683 36 1,282	0.000	-27	3,058 27 2,158	3,120 28 2,079	0.114	-2
Globus pallidus	4,115 63 1,089	5,599 69 1,180	0.000	-27	4,569 46 2,513	4,774 43 2,380	0.001	-4
Dentate nucleus	6,206 121 985	8,233 153 1,019	0.000	-25	6,938 85 2,586	7,942 92 2,731	0.000	-13
Entorhinal cortex	1,822 22 1,971	2,354 33 1,297	0.000	-23	2,129 20 1,765	2,240 20 2,648	0.000	-5
Amygdala	2,309 26 1,705	3,033 28 1,823	0.000	-22	2,619 18 4,482	2,993 22 3,612	0.000	-13
Nucleus basalis of Meynert	6,551 56 3,017	8,372 67 3,162	0.000	-22	8,061 54 4,106	8,320 56 4,142	0.001	-3
Putamen	1,095 12 927	1,316 12 1,037	0.000	-17	972 7 2,024	961 7 2,373	0.274	+1
Caudate nucleus	1,008 13 845	1,194 13 887	0.000	-16	1,020 9 1,516	1,117 8 2,372	0.000	-9
Inferior Olive	3,833 54 680	4,465 76 448	0.000	-14	4,186 40 1,409	3,832 35 1,739	0.000	+9
Magnocellular LGB	4,795 68 865	5,456 77 895	0.000	-12	5,910 54 2,108	5,996 57 1,938	0.277	-1
Parvocellular LGB	2,466 30 967	2,602 31 939	0.001	-5	2,668 21 2,188	2,819 23 2,098	0.000	-5
Substantia Nigra	8,604 61 3,309	9,008 65 3,248	0.000	-5	9,591 48 6,863	9,141 48 6,533	0.000	+5
Cornu Ammonis	3,235 34 1,463	3,362 35 1,633	0.009	-4	3,260 23 3,244	3,563 25 3,099	0.000	-9

LSE, linearized standard error; n, number of neurons measured; LGB, lateral geniculate body.

Table S 2. The difference between the mean volume of neuronal nuclei in autistic and control cohorts

Brain structure	Cohorts of 4- to 8-year-old subjects				Cohorts of > 8-year-old subjects			
	Autism	Control	$p <$	Volume difference (%)	Autism	Control	$p <$	Volume difference (%)
	Mean LSE n	Mean LSE n			Mean LSE n	Mean LSE n		
Nucelus accumbens	163 2 1,319	281 2 1,238	0.000	-42	225 1 2,971	234 1 3,481	0.000	-4
Purkinje cells	393 5 1,077	574 6 1,073	0.000	-32	562 4 2,506	565 4 2,845	0.649	-0.4
Claustrum	234 2 2,483	400 3 2,729	0.000	-42	266 2 2,911	342 2 3,260	0.000	-22
Thalamus	371 5 1,099	643 6 1,267	0.000	-42	440 4 2,222	451 4 2,162	0.067	-2
Globus pallidus	431 6 1,089	628 7 1,113	0.000	-31	490 4 2,548	454 4 2,440	0.000	+8
Dentate Nucleus	303 6 970	503 9 979	0.000	-40	469 5 2,565	408 5 2,713	0.000	+15
Entorhinal Cortex	405 4 1,993	538 6 1,301	0.000	-25	435 3 2,803	446 3 2,722	0.025	-2
Amygdala	431 4 1,708	605 5 1,832	0.000	-29	496 3 4,503	579 4 3,649	0.000	-14
Nucleus basalis of Meynert	476 4 3,037	654 5 3,122	0.000	-27	562 4 4,149	566 4 4,205	0.406	-1
Putamen	181 3 942	246 3 1,078	0.000	-27	227 2 1,993	226 2 2,355	0.691	+0.4
Caudate nucleus	192 3 848	249 4 823	0.000	-23	166 2 1,519	199 2 2,388	0.000	-17
Inferior olive	507 8 670	613 11 428	0.000	-17	552 5 1,431	514 5 1,748	0.000	+8
Magnocellular LGB	389 5 871	465 6 901	0.000	-16	531 4 2,109	501 4 1,984	0.000	+6
Parvocellular LGB	216 3 983	266 3 947	0.000	-19	280 2 2,189	273 2 2,167	0.015	+3
Substantia nigra	756 5 3,348	815 5 3,219	0.000	-7	783 4 6,954	729 4 6,641	0.000	+8
Cornu ammonis	600 5 1,481	713 6 1,630	0.000	-16	644 4 3,269	687 4 3,148	0.000	-6

LSE, linearized standard error; n, number of neurons measured; LGB, lateral geniculate body.

Table S3a. The difference between the mean volume of neuronal soma in autistic and control cohorts in the amygdala nuclei, sectors of the corny Ammonis and entorhinal cortex layers

Cytoarchitectonic subdivisions	Cohorts of 4- to 8-year-old subjects				Cohorts of > 8-year-old subjects			
	Autism	Control	*p* <	Volume difference (%)	Autism	Control	*p* <	Volume difference (%)
	Mean LSE n	Mean LSE n			Mean LSE n	Mean LSE n		
Amygdala, accessory basal nuclei	3,105 55 -	3,883 61 -	0.000	-20	3,590 40 -	4,118 46 -	0.000	-13
Amygdala, basal nucleus	2,521 1 51 -	3,578 58 -	0.000	-30	3,050 32 -	3,527 42 -	0.000	-14
Amygdala, central nucelus	1,398 21 -	1,886 23 -	0.000	-26	1,563 15 -	1,727 17 -	0.000	-10
Amygdala, lateral nucleus	2,162 42 -	2,890 39 -	0.000	-25	2,381 22 -	2,840 27 -	0.000	-16
CA1 sector	2,237 47 383	2,118 38 422	0.058	+6	2,405 31 929	2,499 33 808	0.041	-4
CA2 sector	3,808 71 354	4,210 64 470	0.000	-10	4,049 46 807	4,137 48 765	0.184	-2
CA3 sector	3,549 65 346	3,684 66 354	0.147	-4	3,237 7 43 697	3,564 45 711	0.000	-9
CA4 sector	3,406 64 375	3,518 62 390	0.208	-3	3,449 43 789	4,081 48 795	0.000	-15
Entorhinal cortex, islands	3,138 72 403	3,543 106 265	0.002	-11	3,203 7 53 679	3,393 54 706	0.012	-6
Entorhinal cortex, layer 3	1,817 33 605	2,366 65 325	0.000	-23	1,873 7 27 795	1,931 29 700	0.143	-3
Entorhinal cortex, layer 5	1,685 40 504	2,367 58 362	0.000	-29	2,210 7 38 635	2,349 40 648	0.011	-6
Entorhinal cortex, layer 6	1,345 29 502	1,704 37 356	0.000	-21	1,496 7 23 678	1,655 24 656	0.000	-10

LSE, linearized standard error

Table S3b. The difference between the mean volume of neuronal soma in autistic and control cohorts in four NBM nuclei and three subdivisions of the substantia nigra

Cytoarchitectonic subdivisions	Cohorts of 4- to 8-year-old subjects				Cohorts of > 8-year-old subjects			
	Autism	Control	*p* <	Volume difference (%)	Autism	Control	*p* <	Volume difference (%)
	Mean LSE n	Mean LSE n			Mean LSE n	Mean LSE n		
Nucleus basalis of Meynert Ch1	5,197 125 417	5,615 150 371	0.032	-7	5,680 108 617	5,926 120 463	0.127	-4
Nucleus basalis of Meynert Ch2	5,319 97 633	6,599 117 536	0.000	-19	6,126 99 683	6,323 105 799	0.171	-3
Nucleus basalis of Meynert Ch3	5,999 84 1,021	7.795 113 873	0.000	-23	7,512 87 1,142	7,630 89 1,102	0.341	-2
Nucleus basalis Meynert Ch4	8,562 118 929	10,350 102 1,380	0.000	-17	9,867 84 1,633	10,057 87 1,755	0.117	-2
Substantia nigra, dorsal part	8,507 93 1,257	8,803 101 1,235	0.031	-3	9,229 76 2,802	8,932 76 2,531	0.006	+3
Substantia nigra lateral part	8,881 121 987	9,004 129 954	0.488	-1	9,918 98 1,791	9,595 98 1,846	0.020	+3
Substantia nigra, ventral part	8,610 105 1,074	9,329 110 1,069	0.000	-8	9,624 78 2,257	8,969 77 2,149	0.000	+7

LSE, linearized standard error.

Table S4a. The difference between the mean volume of neuronal nuclei in autistic and control cohorts in the amygdala nuclei, sectors of the cornu Ammonis and entorhinal cortex layers

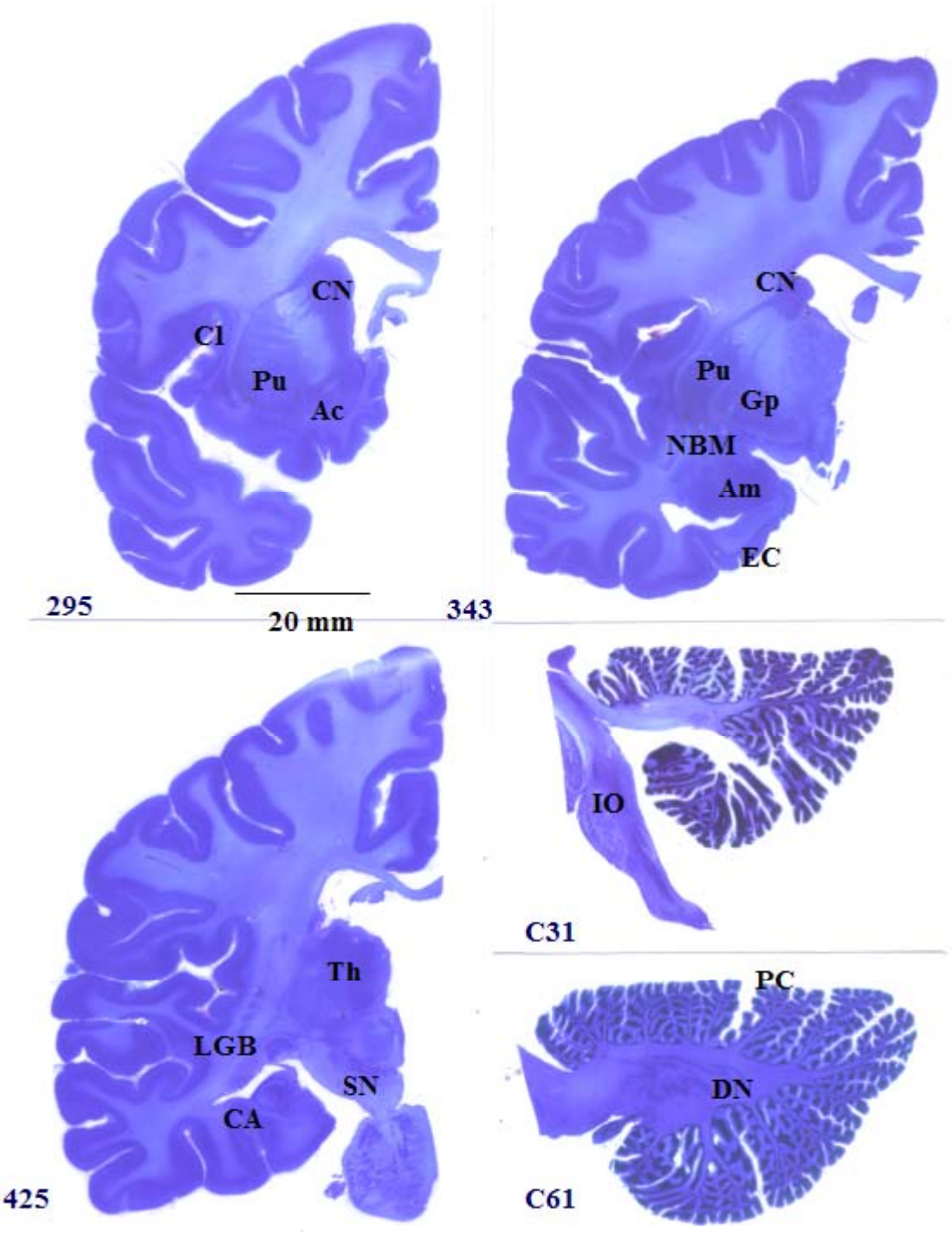
Cytoarchitectonic subdivisions	Cohorts of 4- to 8-year-old subjects				Cohorts of > 8-year-old subjects			
	Autism	Control	*p* <	Volume difference (%)	Autism	Control	*p* <	Volume difference (%)
	Mean LSE n	Mean LSE n			Mean LSE n	Mean LSE n		
Amygdala, accessory basal nuclei	518 9 415	781 11 414	0.000	-34	617 7 1,094	730 8 896	0.000	-16
Amygdala, basal nuclei	467 9 458	682 10 493	0.000	-32	569 6 1,189	666 7 909	0.000	-15
Amygdala, central nuclei	326 5 392	408 5 398	0.000	-20	340 3 1,059	374 4 931	0.000	-9
Amygdala, lateral nuclei	406 7 443	588 7 542	0.000	-31	465 5 1,176	571 6 915	0.000	-18
CA1 sector	441 7 392	522 8 424	0.000	-16	522 6 943	536 5 819	0.085	-3
CA2 sector	672 10 358	821 11 480	0.000	-18	758 8 809	761 8 778	0.801	- 0.4
CA3 sector	661 10 352	747 11 350	0.000	-12	641 7 695	687 7 713	0.000	-7
CA4 sector	648 11 379	783 11 378	0.000	-17	670 7 806	765 7 825	0.000	-12
Entorhinal cortex, islands	524 10 394	633 15 263	0.000	-17	530 7 679	548 7 704	0.063	-3
Entorhinal cortex, layer 3	414 6 604	537 12 325	0.000	-23	396 5 809	396 5 710	0.998	0.0
Entorhinal cortex, layer 5	360 7 499	523 10 356	0.000	-31	446 6 638	447 6 660	0.910	-0.2
Entorhinal cortex, layer 6	365 7 506	469 9 346	0.000	-22	385 6 680	405 6 665	0.011	-5

LSE, linearized standard error.

Table S4b. The difference between the mean volume of neuronal nuclei in autistic and control cohorts in four NBM nuclei and three subdivisions of the substantia nigra.

Cytoarchitectonic subdivisions	Cohorts of 4- to 8-year-old subjects				Cohorts of > 8-year-old subjects			
	Autism	Control	*p* <	Volume difference (%)	Autism	Control	*p* <	Volume difference (%)
	Mean LSE n	Mean LSE n			Mean LSE n	Mean LSE n		
Nucleus basalis of Meynert Ch1	373 8 420	388 10 365	0.214	-4	391 7 618	391 8 472	0.997	-0.0
Nucleus basalis of Meynert Ch2	378 7 645	486 9 527	0.000	-22	454 7 679	420 7 797	0.001	+8
Nucleus basalis of Meynert Ch3	436 6 1,033	584 8 843	0.000	-25	519 6 1,155	495 6 1,130	0.006	+5
Nucleus basalis of Meynert Ch4	640 8 931	838 7 1,368	0.000	-24	681 6 1,650	707 6 1,786	0.002	-4
Substantia nigra, dorsal part	769 8 1,269	777 8 1,235	0.477	-1	764 6 2,868	728 6 2,593	0.000	+5
Substantia nigra, lateral part	724 9 992	804 10 928	0.000	-10	774 7 1,800	713 6 1,858	0.000	+9
Substantia nigra, ventral part	773 9 1,086	876 10 1,054	0.000	-12	808 6 2,282	742 6 2,198	0.000	+9

LSE, linearized standard error.


CN
Cl
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Ac
295
20 mm
CN
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425
PC
DN
C61

Fig. 1

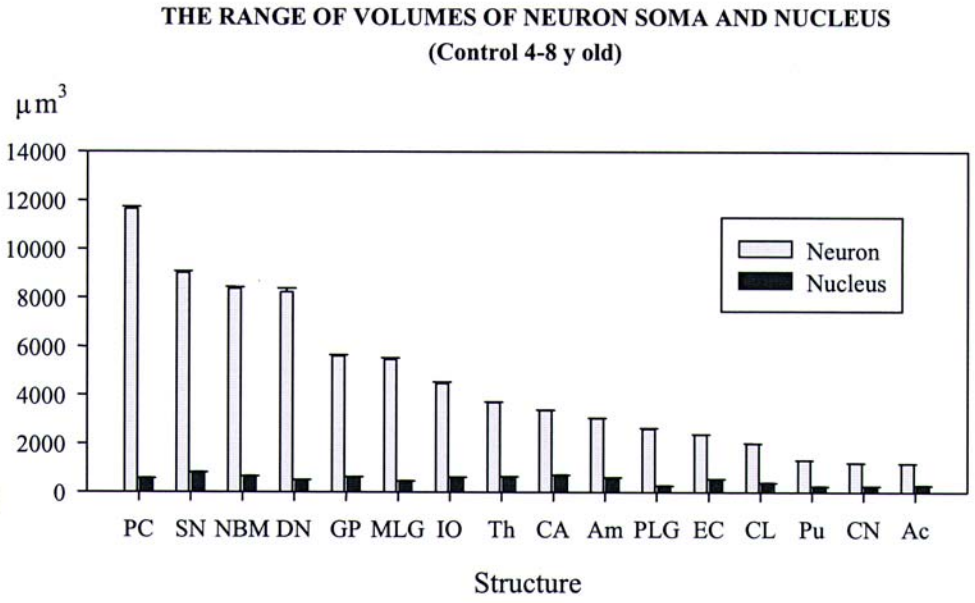
THE RANGE OF VOLUMES OF NEURON SOMA AND NUCLEUS
(Control 4-8 y old)
μm3
14000
12000
10000
8000
6000
4000
2000
0
Neuron
Nucleus
PC SN NBM DN GP MLG IO Th CA Am PLG EC CL Pu CN Ac
Structure

Fig. 2

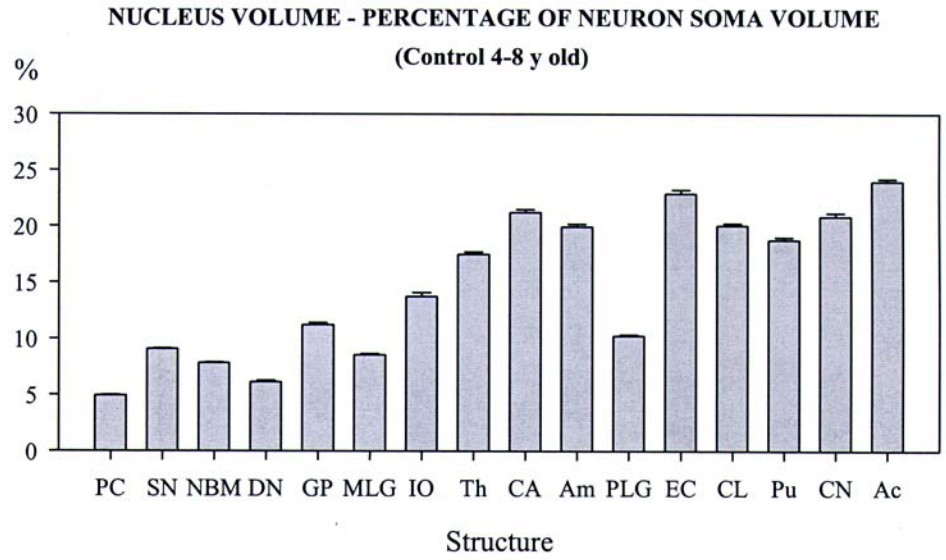
NUCLEUS VOLUME - PERCENTAGE OF NEURON SOMA VOLUME
(Control 4-8 y old)
%
30
25
20
15
10
5
0
PC SN NBM DN GP MLG IO Th CA Am PLG EC CL Pu CN Ac
Structure

Fig. 3

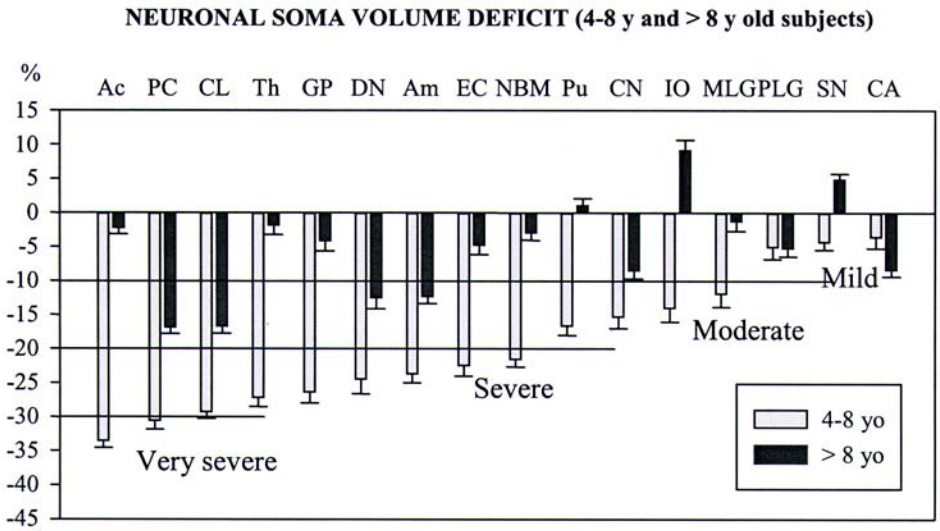
NEURONAL SOMA VOLUME DEFICIT (4-8 y and > 8 y old subjects)
%
Ac PC CL Th GP DN Am EC NBM Pu CN IO MLGPLG SN CA
15
10
5
0
-5
-10
-15
-20
-25
-30
-35
-40
-45
Mild
Moderate
Severe
Very severe
4-8 yo
> 8 yo

Fig. 4

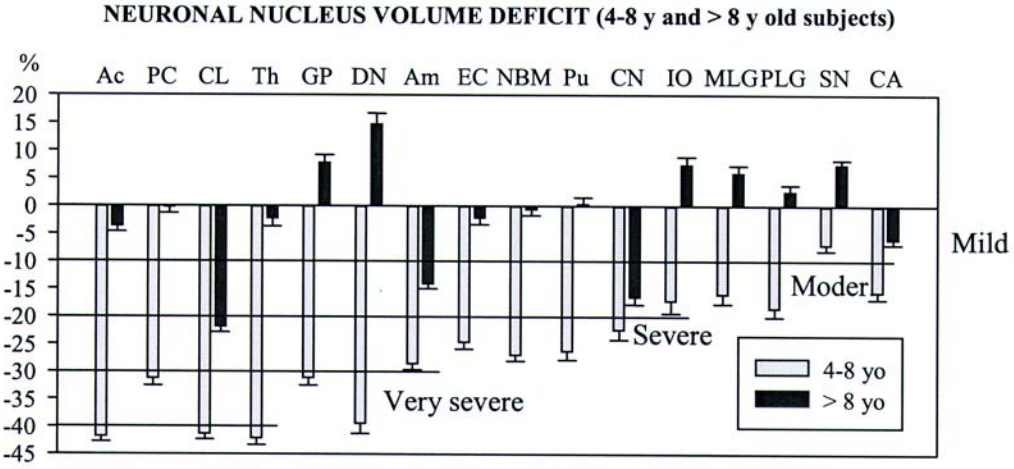
NEURONAL NUCLEUS VOLUME DEFICIT (4-8 y and > 8 y old subjects)
%
Ac PC CL Th GP DN Am EC NBM Pu CN IO MLGPLG SN CA
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-35
-40
-45
Mild
Moder
Severe
Very severe
4-8 yo
> 8 yo

Fig. 5

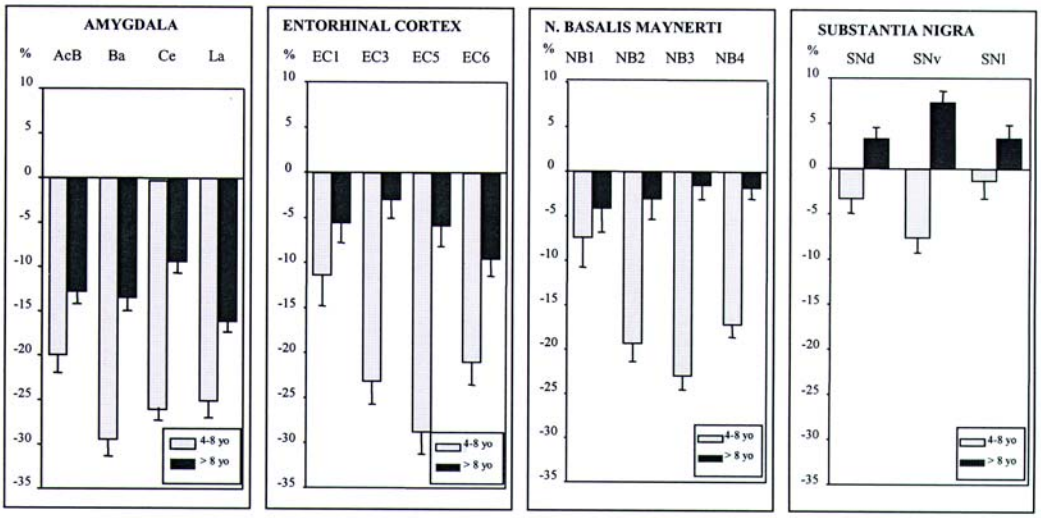
AMYGDALA
% AcB Ba Ce La
ENTORHINAL CORTEX
% EC1 EC3 EC5 EC6
N. BASALIS MAYNERTI
% NB1 NB2 NB3 NB4
SUBSTANTIA NIGRA
% SNd SNv SNl
10
5
0
-5
-10
-15
-20
-25
-30
-35
4-8 yo
> 8 yo

Fig. 6

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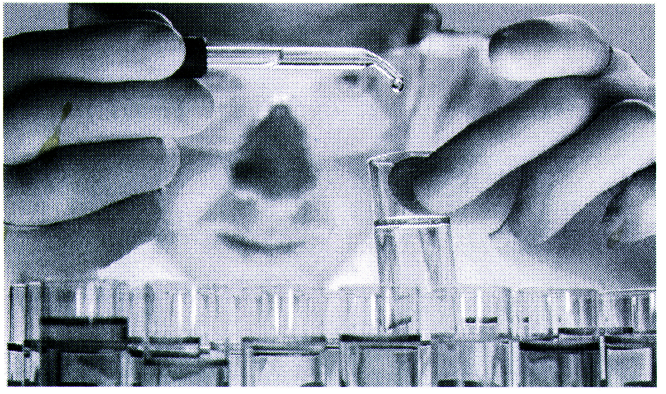
Science Matters: The Latest Advances in Autism Research

New Trends in Brain and Tissue Banking for Autism Research

By Jerzy Wegiel, PhD, Daniel Lightfoot, PhD, Jane Pickett, PhD, and W. Ted Brown, MD, PhD



Professor Giovanni Morgagni, of the University of Padua, published a book in 1761 entitled, "The Seats and Causes of Disease Investigated by Anatomy." This book described nearly 700 autopsies and demonstrated that disease is recorded in the pathology of organs in detectable ways. Dr. Richard Cabot's review of the autopsy records of thousands of patients at the Massachusetts General Hospital in the 1910's revealed that the given clinical diagnosis was wrong in about 40% of cases. These studies, which were also confirmed by many others, justified the central role of autopsies in medical education and in the quality control of clinical practice. They contributed to the growth of autopsy rates in hospitals and medical schools to approximately 50% in the 1940s (Dobbs 2005). This trend resulted in remarkable progress in diagnosis, identification of new diseases, detection of disease mechanisms, and new treatments. However, the rate of autopsies declined toward the end of the past century and the current US rate is less than 5%. The major reasons for declining autopsy rates were costs, changed diagnostic priorities, and decreasing clinical interest in the autopsy as a quality control of clinical diagnosis and therapy (Kretzschmar 2009). The decline in autopsy rates occurred during the same time that the diagnosis rate for Autism Spectrum Disorders (ASD) increased from about 1/2,000 in 1980 to 1/110 in 2010 (The Center for Disease Control). This decline has had a detrimental effect on the progress of research on autism.

Clinical studies of thousands of autistic patients have resulted in improvements in early diagnosis, and behavioral and pharmacological treatments. Genetic studies are identifying gene mutations, single nucleotide polymorphisms, and copy number variations, as contributors to autism's etiology. However, the progress of clinical and genetic studies is not paralleled by a similar progress in postmortem studies of the brain. The brain of an autistic individual is the main source of information about developmental defects determining the cause of life-long disability and clinical phenotypes. The decline in autopsy rates and the number of brain donations for research coincide with an emerging need for application of modern methods of brain studies to determine the types and distribution of developmental alterations, the correlations between structural

see New Trends on page 26

New Trends from page 1

changes and clinical phenotypes, the link between genetic factors and brain structural and functional alterations. It is anticipated that the integration of genetic and clinical studies with neuropathological and biochemical studies of the brain will help in the discovery of the mechanisms that lead to the autistic phenotype and in the design of mechanism-oriented targeted treatments.

Our knowledge of the clinical phenotype and genetic factors in autism is based on the examination of thousands of individuals with autism. However, a review of the world literature revealed that between 1980 and 2003, only 58 brains of individuals with idiopathic autism were examined postmortem (Palmen et al 2004). This low rate of brain donation for research is the major obstacle in research progress. Neuropathological studies are usually limited to a few brains. Due to the very low rate of brain tissue donations for autism research and the etiological and clinical diversity of autism, the pattern of detected changes is incomplete and inconsistent, and neuropathological diagnostic criteria of autism have not yet been established (Lord et al 2000, Pickett and London, 2005).

Research on Autism Requires New Standards of Tissue Banking, Handling, Distribution and Sharing

Brain banking is a particularly important tool for making progress at a time when new technologies in molecular biology, biochemistry and confocal microscopy are opening up new avenues of research on autism. However, autism spectrum disorders are so different than other targets of postmortem studies that they require appropriate research design, and standards of tissue acquisition, preservation and distribution:

1) Autism affects the entire life of an individual with modifications of the phenotype during childhood, adulthood and aging. To detect and characterize developmental and aging associated changes, the cohort examined must represent the entire lifespan and the number of examined individuals must provide statistical power to detect significant differences.

2) Clinical and neuropathological studies reveal a broad spectrum of inter-individual clinical manifestations, most likely as a cumulative effect of genetic and epigenetic factors determining both structural and functional alterations. This heterogeneity requires large enough cohorts to identify and characterize major autism phenotypes and mechanisms shaping these phenotypes.

3) Autism is diagnosed in association with other syndromes/disorders, including fragile X syndrome, chromosome 15 duplication, Down syndrome, seizures, and intellectual deficits. It requires parallel studies of reference cohorts.

4) Qualitative developmental abnormalities are usually undetectable by routine neuropathological examination. However, modification and expansion of neuropathological evaluation methods reveals a broad spectrum of defects of neurogenesis, migration and cytoarchitecture (Wegiel et al 2010a). A majority of developmental changes are mainly quantitative and only unbiased morphometric methods can detect significant changes of brain development.

5) Changes in the developing and the aging brain of autistic individuals have a global character with brain region, neuronal population, neuronal circuit and neurotransmitter system specific alterations. Therefore research on autism requires the combining of localized models into global models of brain developmental defects, keeping in mind that each autistic patient is unique and all statistical strategies are dealing with heterogeneity.

Age Associated Alterations in the Brain of People Diagnosed with Autism

In contrast to majority of human illnesses, autism is a life-long disability with age-specific alterations. The global marker of age-associated alterations is abnormal acceleration of brain growth in autistic children 1 to 2 years of age (Courchesne et al 2001, 2003, Dawson et al 2007), slower rate of brain growth at age of 2 to 4 years (Courchesne et al 2001, Hazlett et al 2005), and a decrease to control levels in the middle to late childhood period. The period of accelerated brain growth rate precedes and overlaps with the onset of detectable behavioral changes, and the period of deceleration coincides with worsening of autism symptoms (Dawson et al 2007). Recent data indicate that these changes are associated with an increase of the total number of neurons (Courchesne et al 2011). Our studies reveal that in the majority of examined brain regions, the volume of neurons is less than in normally developing children, but these differences are almost undetectable in the brains of teenagers/adults (Wegiel et al 2010). Identification of the self-regulatory mechanisms that can lead to brain size and neuron size normalization in early childhood may result in treatments that reduce or eliminate developmental delay and restore correct trajectory of brain development and function. Altered brain development suggests that the aging autistic brain will be also modified, but late age-associated changes are almost unexplored.

Brain Only?

Major research efforts are focused on the brains of autistic individuals. However, clinical records suggest that immune, digestive and peripheral nervous system alterations are also present in autism. One abnormality is of particular interest. Hyperserotonemia in autism, identified as an increase in the serotonin level in blood platelets by up to 50% is a frequent finding. A significant amelioration of obsessive-compulsive rituals and routines, and anxiety and aggression in subjects with autism treated with selective serotonin reuptake inhibitors, such as fluoxetine (deLong et al 1998, Kolevzon et al 2006) confirms the hypothesis that the serotonergic system is altered and that modulation of these developmental alterations produces clinical improvements.

Blood serotonin is produced by enterochromaffin cells of the intestinal epithelium whereas brain serotonin is produced by neurons of the raphe nuclei projecting from the brainstem to all cortical and subcortical subdivisions. However, in spite of evidence (a) that the development of the serotonergic system of autistic individuals is altered, (b) that these alterations contribute to the clinical manifestations of autism, and that (c) pharmacological interventions result in significant benefits of some autistic patients, enterochromaffin cells of the intestines, the source of blood serotonin, and the raphe nuclei, the only source of serotonin supporting the entire brain function and contributing to autistic phenotype were not examined. These examples of gaps in our knowledge of the pathology of autism indicate that changes in standards of tissue preservation for research are necessary.

The Role of Brain and Tissue Banks

One of factors contributing the low rate of tissue donation is the limited knowledge of the general public about legal and technical aspects of donation as well as the important role of postmortem studies of the brain and other organs in research progress on autism. Banks play a critical role in cooperating with families and in obtaining clinical records. Brain and tissue banks services for families are free of any charges to them. Tissue samples as well as clinical data are distributed to qualified researchers in a coded and anonymous form. The next of kin, who signs the donation document, is eligible to receive a neuropathological report based on examination of the brain and histopathological sections, also without charges. To match to the complexity of modern research, the banks adopt complex protocols of tissue collection, clinical data acquisition, clinical and neuropathological diagnosis, tissue quality evaluation, tissue preservation, processing, storage, and distribution. One brain provides hundreds of tissue samples dissected with anatomical precision to obtain information about specific brain structures or neuronal populations and samples are distributed to dozens of projects (Vonsattel et al 2008). Estimates of the brain bank cost per brain preserved for postmortem studies is in range between $10,000 to $30,000 in US and 10,000 to 15,000 euros for the BrainNet Europe consortium (Hulette 2003). However, brain and tissue banks efforts reduce the risk of inclusion in dozens of research projects and publications a case with an incorrect genetic, clinical, neuropathological classification or affected by postmortem degradation distorting biochemical and neuropathological studies. As a result, tissue banks contribute to elimination of false results, increased quality of research and reduce the costs of the individual research project. Autism tissue banking is supported by the Autism Speaks/Autism Tissue Program and new initiatives of the New York State Autism Consortium.

Autism Tissue Program

In response to both families and researchers, requests to the Autism Tissue Program of Autism Speaks provides information and support for families donating tissue for research and concentrates on enhancing the availability of brain tissue for research (www.autismtissueprogram.org). To advance research on autism and related disorders, ATP coordinates tissue recovery, storage, cataloging, preservation, and distribution of brain and other tissues to qualified investigators. The tissue collection includes samples from normal individuals (a control cohort) and individuals with developmental disabilities other than autism.

New York State Autism Consortium

New York State Office for People with Developmental Disabilities (OPWDD) supports more than 16,000 people diagnosed with autism and ASD, and the number of autistic subjects increases every year.. In response to the growing autism crisis, in 2008, the OPWDD created a New York State Autism Consortium under the leadership of the Institute for Basic Research in Developmental Disabilities (IBR). The aim of the Consortium is to establish the infrastructure, resources and collaboration necessary to advance basic and applied research on autism. The consortium collaborates with affected families, advocacy organizations, research institutions and public agencies to implement new research on the causes, mechanisms and treatment of autism. One of tasks of Consortium is support and expansion of tissue donation and banking, including brain, and other tissues and organs.

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W. Ted Brown, MD, PhD, is the Director of the New York State Institute for Basic Research in Developmental Disabilities and its George A. Jervis Clinic located on Staten Island, New York.

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see New Trends on page 29

New Trends from page 26

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